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THE CAUSE OF THE DISAPPEARANCE OF ARGININE FROM THE BLOOD OF RATS WITH ACUTE HEPATIC NECROSIS INDUCED BY DIETARY MEANS[1]

GUY N. EMERY AND J. M. R. BEVERIDGE

Abstract

One hundred and fifteen male weanling rats of the Sprague–Dawley strain were placed on a basal ration designed to deplete their tissue reserves of vitamin E and selenium. They were then placed on a diet capable of precipitating acute hepatic necrosis within a few days. At 0, 12, 24, 36, 48, 60, 72, 78, 84, 90, 96, 114, 120, 132, 144, and 160 hours, groups of rats, including any which showed obvious signs of liver damage, were anesthetized with pentobarbital and blood samples were taken from the aorta via a syringe. Determinations of arginase activity of the plasma showed that greatly elevated values occurred only at the time of development of the acute liver damage. It is concluded that the disappearance of arginine from the blood of rats in which acute liver necrosis has developed is due to the liberation of arginase from the dead and dying liver cells thus destroying the amino acid.

In a recent study of the amino acid pattern of the blood of rats with acute hepatic necrosis induced by dietary means, Valberg and Beveridge (1) found that although arginine was present in the blood of normal animals and in those on a necrogenic diet prior to the development of the liver lesion, it was not detectable in those animals in which the acute liver lesion was present. There were two rather obvious possibilities that might serve to explain this phenomenon: the release of arginase from dead and dying liver cells, a tissue especially rich in this enzyme, and (or) a renal lesion permitting a preferential loss of arginine in the urine. The latter possibility was held to be the less likely of the two explanations despite the fact that a frank morphological lesion in the renal tubules often occurs at the time of the acute liver damage. The level of arginase activity in the plasma of animals on a diet designed to induce acute liver necrosis was therefore investigated.

[1]Manuscript received January 20, 1961.
Contribution from the Department of Biochemistry, Queen's University, Kingston, Ontario. This investigation was financed by a grant-in-aid from the National Research Council of Canada.

Experimental

One hundred and fifteen male weanling rats of the Sprague–Dawley strain were placed on the following diet designed to deplete their tissue reserves of vitamin E and selenium, either one of which can prevent the hepatic damage (2): alcohol-extracted baker's yeast, 40%; sucrose, 22.5%; vitamin-E-stripped corn oil, 30%; cod liver oil, 1%; L-cystine, 0.25%; DL-methionine, 0.25%; salt mixture, 3% (3); vitamin mixture, 0.7% (4); celluflour, 2%; and choline chloride, 0.3%. After the dialuric acid test became positive, indicating that the animals had become deficient in vitamin E (5), two animals were placed at intervals on a diet modified by removal of the supplements of the sulphur-containing amino acids and by a decrease of the level of yeast from 40 to 18% and a concomitant increase in sucrose. Previous work (6) had demonstrated that after animals consumed the basal diet for a certain length of time, transfer to the foregoing modified regimen can precipitate lethal liver damage within 2 or 3 days. When the test animals were found to die within 4 days, the entire group, which by this time numbered 86, was then placed on the modified ration. At 0, 12, 24, 36, 48, 60, 72, 78, 84, 90, 96, 114, 120, 132, 144, and 160 hours, groups of animals, including any which showed obvious signs of liver damage, were anesthetized with pentobarbital, and blood samples were obtained from the aorta by means of a needle and syringe containing heparin. Of the 86 animals, nine were found dead and five others died due to the anesthetic. Because of these deaths, only 72 samples of blood were obtained. These were centrifuged shortly after collection and the plasma was stored in the deepfreeze for assay of arginase activity at a later date. Arginase has been shown to be stable for months under these conditions (7). The method of Manning and Grisolia (8) with minor modifications was used for the determination of arginase activity. For the purposes of expressing the results reported in this communication, a unit of arginase activity was taken to be that amount which, in 30 minutes at 38° C with a substrate concentration of 0.285 *M* arginine at pH 9.0, will liberate 2.5 mg urea nitrogen.

Results and Discussion

Of the 72 samples of blood obtained, 71 were analyzed in duplicate for arginase activity. Figure 1 shows a close correlation between plasma arginase activity and incidence of necrosis. Twenty-seven animals showed unequivocal evidence of acute massive hepatic necrosis as determined by gross and microscopic examination and in 26 of these the plasma arginase activity was highly elevated. The mean and standard deviation of these were 24 ± 5.6 units per 100 ml plasma. In a number of cases the true arginase activity was much higher than that recorded but repeat estimations on appropriately diluted samples could not be done and the values were simply recorded as 28+ units per 100 ml plasma.

Eight animals showed some elevation of arginase activity although their livers did not show clear-cut microscopic evidence of necrosis. However, in

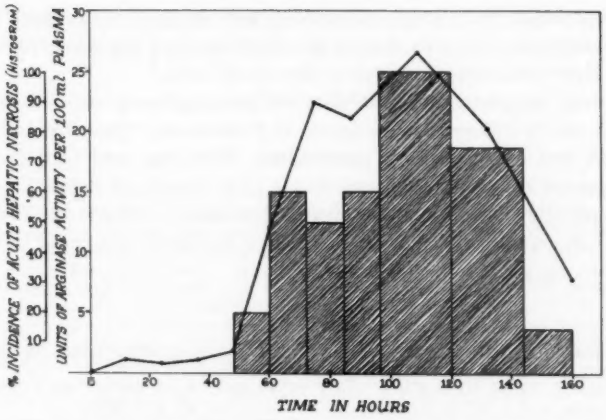

FIG. 1. The continuous line shows the change in the average arginase activity of the plasma of rats depleted of vitamin E and selenium and placed on a ration designed to produce acute hepatic necrosis within 2 or 3 days. The histogram shows the percentage of the animals killed at the times indicated in which acute liver damage was found.

two of these, other signs were present, e.g. yellowish staining around the urethral meatus and abnormally large liver weight (over 6% of body weight). The mean arginase activity and standard deviation of those not showing signs of acute hepatic necrosis (42 rats) were 3.12 ± 5.29 units per 100 ml plasma. The large standard deviation was due to the fact that the arginase level in a few of these animals was elevated but they could not be classed as having acute hepatic necrosis on the basis of the morphological examination. Undoubtedly these animals, had they been permitted to live a little longer, would have shown frank signs of hepatocellular damage. For purposes of comparison, determinations were performed on eight normal animals of the same age and strain maintained on fox chow. The mean and standard deviation were 0.45 ± 0.69 units per 100 ml plasma.

In animals on a dietary regimen such as the one used in this work and particularly at the time of development of the acute liver damage, the red blood cells show increased fragility. This circumstance made it difficult to obtain samples free from hemolysis, a feature that acquired significance in the light of the fact that small amounts of arginase have been reported to occur in erythrocytes (9). In order to check on the degree of interference that hemolysis might introduce, eight samples of blood from normal rats were divided into two aliquots each. One of each pair was hemolyzed by addition of distilled water and arginase activity was determined on all samples. The values were essentially identical and it was concluded that the slight degree of hemolysis in some of the blood samples of the test series of animals did not contribute significantly to the observed arginase activity.

There is little doubt therefore that the disappearance of arginine from the

blood of rats which had developed acute liver necrosis as previously reported from our laboratory (1) was due to the liberation of arginase from the dead and dying liver cells thus destroying the amino acid.*

This finding suggests the possibility of using plasma arginine or arginase levels as an aid in differential diagnosis in those cases where liver cell damage is suspected but not definitely established. Manning and Grisolia (8) have already reported on serum arginase levels in a variety of conditions affecting the liver, and they found slightly elevated values. It would be interesting in the light of the work reported here to perform a study on those patients suspected of having gross hepatocellular damage.

References

1. L. S. VALBERG and J. M. R. BEVERIDGE. Can. J. Biochem. and Physiol. **38**, 365 (1960).
2. K. SCHWARZ and C. M. FOLTZ. J. Am. Chem. Soc. **79**, 3292 (1957).
3. M. R. ABELL, J. M. R. BEVERIDGE, and J. H. FISHER. Arch. Pathol. **50**, 1 (1950).
4. J. R. MCLEAN and J. M. R. BEVERIDGE. J. Nutrition, **47**, 41 (1952).
5. C. ROSE and P. GYORGY. Am. J. Physiol. **168**, 414 (1952).
6. L. S. VALBERG, R. A. YOUNG, and J. M. R. BEVERIDGE. Can. J. Biochem. and Physiol. **37**, 493 (1959).
7. D. M. GREENBERG. Methods of Enzymology, **7**, 368 (1955).
8. R. T. MANNING and S. GRISOLIA. Proc. Soc. Exptl. Biol. Med. **95**, 225 (1957).
9. D. D. VAN SLYKE and R. M. ARCHIBALD. J. Biol. Chem. **165**, 293 (1946).

*Recently we have examined the urine from rats with acute hepatic necrosis and no arginine was detected.

STEADY-STATE OXIDATION OF GLUCOSE IN HYPERTHYROID AND HYPOTHYROID RATS[1]

DOROTHY S. DOW AND C. E. ALLEN

Abstract

The single intraperitoneal injection of a labelled glucose load has been shown to maintain a steady state with respect to the specific activities of blood glucose and expired CO_2 for extended periods of time in hyperthyroid and hypothyroid rats. Measurements were made of rates of oxidation of glucose-1-C^{14} and glucose-6-C^{14} during the steady state.

The results indicate that glucose oxidation by way of the glycolytic pathway is markedly elevated in the hyperthyroid rat and parallels the increased rate of CO_2 expiration. Glucose oxidation by way of the hexose monophosphate pathway is completely suppressed. This effect is considered to be due to the preferential maintenance of diphosphopyridine nucleotide in the reduced form.

In the hypothyroid rat a decreased rate of glucose oxidation by way of both oxidative pathways parallels the decreased rate of CO_2 production.

The results support the view that the primary action of thyroxine is on oxidative activity. The observed effect on the hexose monophosphate pathway appears to be secondary.

Introduction

Rate of oxidation is controlled by rate of phosphorylation in the normal animal but in the animal treated with excess thyroxine this control no longer exists and respiration is elevated dependent upon the level of thyroxine employed (1). Mitochondrial swelling (2) and reduction in the mitochondrial complement of total diphosphopyridine nucleotide (3) precede the uncoupling action of thyroxine on oxidative phosphorylation. However, these phenomena are insufficiently general to account for the effects of thyroxine in vivo since an effect of the hormone was observed in vitro in cells not known to have mitochondria (4). Competition of thyroxine with DPN for zinc sites on dehydrogenases has been suggested as the mechanism of action of thyroxine (5). Thyroxine has been shown to chelate a variety of cations (6) but the difficulty in differentiating between primary and secondary effects of these cations has prevented definition of a possibile chelating role of the hormone in its metabolic activity.

An inhibitory effect of thyroxine has been reported for the transhydrogenase which catalyzes the generation of balanced supplies of pyridine nucleotides (7). Inhibition of this enzyme should either retard processes requiring TPN* or should effect an increase in rate of oxidation of the accumulated TPNH through the mediation of the specific TPNH – cytochrome *c* reductase. Since the latter

[1]Manuscript received December 9, 1960.
Contribution No. 56, Animal Research Institute, Research Branch, Canada Department of Agriculture, Ottawa, Ontario.

*The following abbreviations are used: DPN, diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide; EMP, Embden–Meyerhof pathway; HMP, hexose monophosphate pathway.

pathway is not associated with phosphorylation (8), uncoupling of oxidation from phosphorylation would result. The specific increase in activity of TPNH – cytochrome *c* reductase found in hyperthyroid rat liver appears to confirm an effect of thyroxine on shunting energy away from phosphorylation by this means (9). However, well-washed mitochondria from rats treated with physiological doses of thyroxine exhibited an increase in TPNH – cytochrome *c* reductase activity but not the expected dissociation of oxidation from phosphorylation (10).

The increase in TPNH – cytochrome *c* reductase should also result in increases in metabolic processes proceeding over TPN-linked oxidation, such as the HMP for glucose-6-phosphate utilization. Studies with liver have shown no effect of thyroxine on $C^{14}O_2$ production via the HMP (11) in spite of increased activity of the enzymes of this pathway (12). However, equal stimulation by thyroxine of both the EMP and the HMP in the intact rat has been reported, based on rate of $C^{14}O_2$ production (13).

It has been suggested that the efficiency of oxidation of TPNH through its specific cytochrome *c* reductase may be a rate-limiting step (14). Also, it is uncertain that mitochondria, in general, possess a direct means of oxidizing TPNH (15). If the direct oxidation of TPNH is normally limiting then the observed effects of thyroxine should be consistent with an effect of the hormone on the rate of TPNH–DPN transhydrogenase activity. Under conditions of a deficiency of electron acceptors for TPNH, thyroxine should have a depressant effect on HMP activity.

In order to clarify this point glucose oxidation of both hyperthyroid and control rats was measured during a steady state between the specific activities of the blood glucose and the expired CO_2. Steady-state glucose oxidation of hypothyroid rats was also determined.

Materials and Methods

The test animals were male albino rats of the Wistar strain. Treated rats and their corresponding controls were within the same weight ranges. The basal diet was powdered fox cubes. All diets and drinking water were offered ad libitum.

The thyroxine diets contained the hormone in amounts varying from 0.007 to 0.009% as Na-*l*-thyroxine. Severe hyperthyroidism was considered to have been established when animals on these diets failed to gain.

The goitrogenic diets contained 0.1% thiouracil. The criteria of goitrogenicity were reduced rate of body weight gain and gross hypertrophy of the thyroid gland.

Different groups of control, hyperthyroid, and hypothyroid rats were used for separate collections of blood and expired CO_2 following the intraperitoneal injection of a labelled glucose load. The injected solutions, which were of similar specific activity, contained either glucose-1-C^{14} or glucose-6-C^{14} in 5% unlabelled aqueous glucose. The injected dose contained 150 mg glucose; the

total radioactivity administered was approximately 3.5×10^6 c.p.m. as measured in a windowless gas-flow counter. The specifically labelled isotopes were obtained from Merck and Co. Ltd., Montreal. Blood glucose concentration and rate of expired CO_2 production were measured, as well as their specific activities, at regular time intervals after glucose-C^{14} administration.

Measurement of Specific Activity of Blood Glucose

Following injection of the labelled glucose load, separate groups of three rats each were bled individually at 15-minute intervals up to a period of 2 hours. A brief warming of the rat ensured the securing of 0.5 ml of blood from the tail vein. Blood glucose concentration was determined by the method of Mendel *et al.* (16).

Purified osazones were prepared from the blood samples as described by Dow and Allen (17), and were transferred to aluminum planchets for measurement of radioactivity. Corrections for self-absorption were applied according to Karnovsky *et al.* (18). Melting point determinations indicated a satisfactory degree of purity of the phenylosazones.

Measurement of Specific Activity of Expired Carbon Dioxide

The respiration apparatus used was that described by Gidez (19). Dry, CO_2-free air was drawn through the system by a vacuum pump at a rate controlled by a flowmeter and diffused through a fine mesh grid beneath the platform supporting the animals. Silica gel prevented the accumulation of moisture in the animal chamber. Two sets of interchangeable gas-washing bottles containing NaOH allowed the removal of samples without interruption of the gas flow. A terminal bottle containing $Ba(OH)_2$ indicated the efficiency of CO_2 absorption.

Immediately after receiving an intraperitoneal injection of radioactive glucose, two or three rats, depending on the anticipated CO_2 production, were placed in the respiration chamber, and samples were removed at 30-minute intervals. No samples were collected for the initial 30-minute interval.

Carbon dioxide was recovered as $BaCO_3$. Total carbonate values were obtained either by titration or by the Van Slyke manometric technique, and these were in good agreement. Pads were prepared by filtration on filter paper, then transferred to plastic disks for counting. Corrections were made for self-absorption.

Results

The data in Table I show that blood glucose concentration of both hyperthyroid and hypothyroid rats is less than that of untreated controls. Although the injection of a 150-mg sugar load has had a variable effect on blood glucose concentration it has not resulted in a persistent hyperglycaemia in any of the treatment groups.

Table II shows representative values for expired CO_2 for hyperthyroid and hypothyroid rats. As might be expected from the known actions of thyroxine and thiouracil on rate of oxidation, the hyperthyroid rats have shown a greater

TABLE I

Effect of intraperitoneal injection of a 150 mg glucose load on blood glucose levels

No glucose load			Time interval after injection of glucose load (hours)	150 mg glucose load		
Control	Hyper-thyroid	Hypo-thyroid		Control	Hyper-thyroid	Hypo-thyroid
Blood glucose (mg%)				Blood glucose (mg%)		
125	82	80	0.25	167	122	150
116	86	78	0.25	165	132	160
116	82	80	0.25	155	117	152
117	86	70	0.50	145	118	143
112	91	78	0.50	135	125	150
127	80	75	0.50	143	127	112
130	83	72	0.75	135	127	128
135	82	75	0.75	145	130	150
123	89	75	0.75	130	127	133
127	76	82	1.00	130	122	112
	80	82	1.00	132	120	105
	100	82	1.00	143	127	130
	87	75	1.25	132	118	110
	82	100	1.25	135	120	125
	100	96	1.25	130	117	120
	82	75	1.50	130	98	115
	72	85	1.50	127	110	117
	82	75	1.50	143	97	120
	80	75	1.75	125	107	112
	86	75	1.75	132	105	110
	81	86	1.75	125	98	117
	72	86	2.00	132	113	107
	66	78	2.00	125	120	110
	89	70	2.00	125	98	107

TABLE II

Effect of thyroxine and thiouracil on CO_2 expiration following intraperitoneal injection of a labelled glucose load

Time interval after injection (hours)	Average body weight							
	Control		Hyperthyroid		Control		Hypothyroid	
	110 g	100 g	108 g	119 g	143 g	143 g	141 g	146 g
	μmoles CO_2/100 g/30 minutes							
0.5–1.0	2800	3200	6663	7325	2471	2317	2295	2434
1.0–1.5	2800	3352	7612	7569	2510	2398	2071	2541
1.5–2.0	3077	3453	6752	8479	2716	3053	2677	2246
2.0–2.5	3213	3357	7140	6556	3284	2485	2623	2434
2.5–3.0	3375	3561	6757	7959	2407	2769	2283	2508
3.0–3.5	2921	3086			3090	2796	2279	2508
3.5–4.0	3391	3170			2239	2550	2505	2305
4.0–4.5	3188	2950			2353	2498	2071	2684
4.5–5.0	3275	3363			3216	2498	2381	2246
0.5-hour av.	3116	3277	6985	7578	2699	2615	2354	2435

NOTE: Each column represents the average production of three rats at successive time intervals. The mean difference between hypothyroid and control is statistically significant ($P < 0.01$).

than twofold increase while the hypothyroid rats have shown a 10% ($P < 0.01$) reduction in CO_2 production. Since the injection of a glucose load did not consistently affect blood glucose level and since CO_2 production did not vary significantly throughout the test period in any of the treatment groups it can be concluded that rate of CO_2 production was not influenced by the sugar load.

Previous trials (17) showed that the intraperitoneal injection of a labelled glucose load into both diabetic and control rats maintained the specific activities of both blood glucose and expired CO_2 at constant levels for extended periods of time. It is apparent in Figs. 1–4 that plateaus in the specific activities of blood glucose and expired CO_2 have also been established in hyperthyroid and hypothyroid rats under similar conditions. It is concluded that a steady state between the two components has been established in treated and control rats. The data illustrated in Figs. 1 and 2 indicate that the steady-state value for the

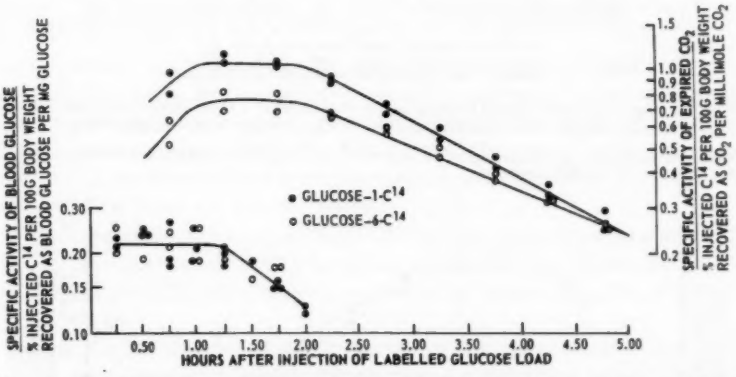

FIG. 1. Specific activities of blood glucose and expired CO_2 of control rats following intraperitoneal injection of a labelled glucose load. Average body weight, 109 g.

specific activity of blood glucose has been lowered from 0.22 to 0.15% in the hyperthyroid rat in comparison with the control of the same weight. A release of unlabelled sugar due to gluconeogenesis could account for this effect since hyperthyroidism has been shown to accelerate the biosynthesis and metabolism of adrenal glucocorticoids (20, 21). Identical steady-state values for expired CO_2 following both glucose-1-C^{14} and glucose-6-C^{14} in the hyperthyroid rat indicate no difference between the isotopes in rate of utilization. The fact that the steady-state level for CO_2 is similar to that for the control for glucose-6-C^{14}, although the steady-state level for glucose is reduced, would appear to indicate a slight preference for oxidation of glucose in the hyperthyroid rat. The fact that the specific activity of the expired CO_2 in the hyperthyroid rat reached a steady-state value earlier than did the control would appear to indicate an increased rate of oxidation of glucose. Figures 3 and 4 show that the steady-state values for both blood glucose and expired CO_2 for the hypothyroid rat are

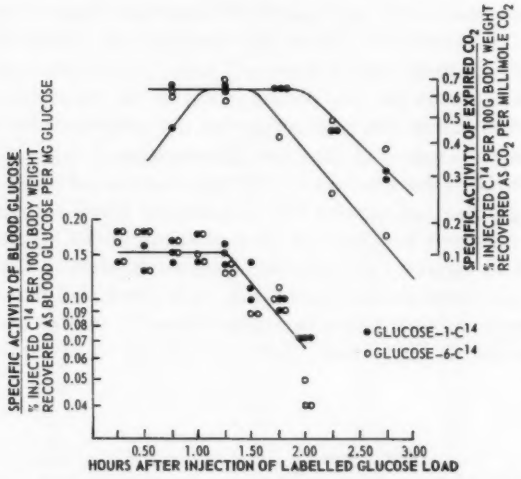

FIG. 2. Specific activities of blood glucose and expired CO_2 of thyrotoxic rats following intraperitoneal injection of a labelled glucose load. Average body weight, 95 g.

NOTE: Plateaus in the specific activity of the expired CO_2 persisted for at least 1 hour following the injection of both glucose-1-C^{14} and glucose-6-C^{14}.

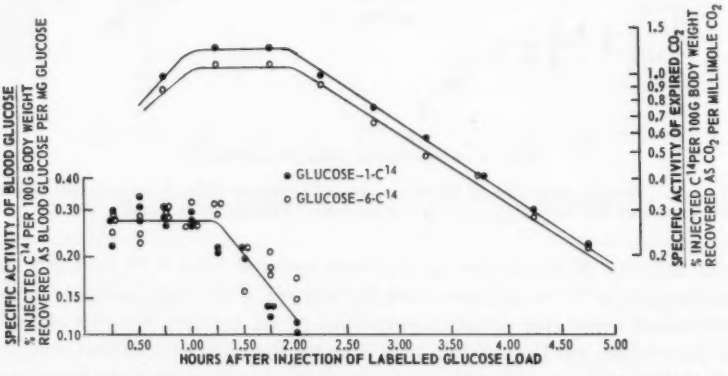

FIG. 3. Specific activities of blood glucose and expired CO_2 of control rats following intraperitoneal injection of a labelled glucose load. Average body weight, 150 g.

not different from those for the control of the same weight. The steady-state value for blood glucose for the hypothyroid rat does not indicate an effect of treatment on gluconeogenesis although an inhibitory effect of hypothyroidism on the biosynthesis and metabolism of glucocorticoids has been reported (20, 21). The fact that the steady-state value for blood glucose in the hypothyroid rat persisted longer than in the control would appear to indicate a reduced rate of oxidation of glucose.

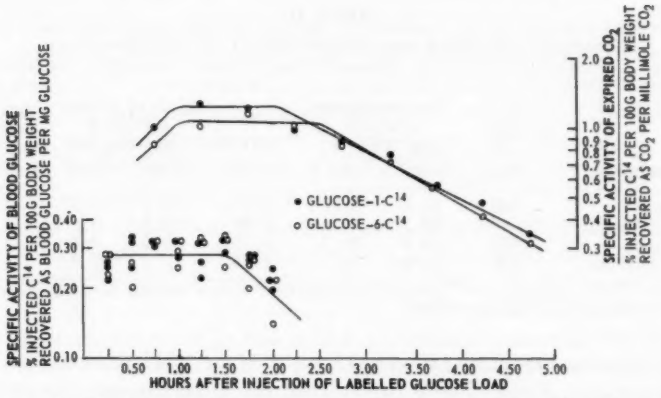

FIG. 4. Specific activities of blood glucose and expired CO_2 of hypothyroid rats following intraperitoneal injection of a labelled glucose load. Average body weight, 150 g.

Since a steady state between the specific activities of blood glucose and expired CO_2 has been established in all treatment groups, it should be valid to assume that blood glucose is being oxidized directly to CO_2 at constant rates while the plateaus persist. With this assumption, the rates of oxidation of body glucose to CO_2 were calculated from the specific activity – time curves of blood glucose and CO_2 thus: $(A \times B)/C$, where

A = specific activity of expired CO_2 at the plateau, expressed as % of the injected C^{14}/100 g body wt. recovered as CO_2/mmole CO_2,

B = rate of CO_2 production, expressed as mmole CO_2/100 g body wt./hour,

C = specific activity of blood glucose at the plateau, expressed as % of the injected C^{14}/100 g body wt. recovered as blood glucose/mg glucose.

The percentage labelling of the blood glucose was found to decrease with increasing body weight following a constant dose of labelled glucose. (This effect was demonstrated in groups of untreated rats ranging from 50 to 175 g body weight.) Consequently, in order to correct for the effect of body weight variation, the specific activity values are presented as a percentage of the injected C^{14} per 100 g body weight recovered per unit of the isolated component.

The calculations presented in Table III are based on the widely accepted assumption that carbons 1 and 6 are metabolized to CO_2 at the same rate by way of the glycolytic cycle whereas carbon 1 is preferentially metabolized to CO_2 by way of the hexose monophosphate pathway. This assumption seems reasonable for a relatively short time interval after the injection of the labelled glucose. Recycling of C^{14} is considered to be insignificant during the steady state that has been established.

Table III shows glucose oxidation rates for hyperthyroid and hypothyroid rats and for controls of similar body weights. The data for the younger controls

TABLE III

Steady-state oxidation rates of hyperthyroid and hypothyroid rats

Treatment	Av. body wt. (g)	Rate of glucose oxidation (mg/100 g/hr) G-1-C^{14}	Rate of glucose oxidation (mg/100 g/hr) G-6-C^{14}	G-1-C^{14} / G-6-C^{14}	Rate of glucose oxidation (mg/100 g/hr) EMP	Rate of glucose oxidation (mg/100 g/hr) HMP	CO_2 derived from glucose (%)
Control	109	31.4	22.2	1.41	22.2	9.2	16.4
Hyperthyroid	95	58.1	58.1	1.00	58.1	0	13.4
Control	150	23.9	20.5	1.17	20.5	3.4	15.0
Hypothyroid	150	21.0	18.1	1.16	18.1	2.9	14.6

NOTE: G-1-C^{14} = glucose-1-C^{14}; G-6-C^{14} = glucose-6-C^{14}; EMP = Embden–Meyerhof pathway; HMP = hexose monophosphate pathway.

show that glucose oxidation proceeded by way of the EMP at better than twice the rate by way of the HMP. In contrast, the hyperthyroid rats showed an increase in glucose oxidation by way of the EMP which was comparable to the increase in total CO_2 production and there was no glucose oxidation by way of the HMP. The calculated percentage of the total CO_2 which was derived from glucose reflects the inhibition of the HMP. For the older controls, whose growth rate had begun to decline, the data show a marked reduction in the rate of glucose oxidation by way of the HMP in comparison with the younger controls. This finding is in agreement with other reports that growth is promoted by the supply of pentose phosphate (22). The data for the goitrogenic rat show that a reduction in glucose oxidation by way of both oxidative pathways paralleled the reduction observed in total CO_2 production.

Discussion

The intraperitoneal injection of a labelled glucose load into control, hyperthyroid, and hypothyroid rats has resulted in persistent plateaus in the specific activities of blood glucose and expired CO_2. Conflicting evidence has been presented with regard to hyperglycaemia as the causative factor for a plateau in blood glucose specific activity (23, 24). Since there was no consistent pattern of hyperglycaemia in control, hyperthyroid, or hypothyroid rats in these trials, this condition does not appear to have been the causative factor for the observed plateaus. The formation of a plateau must involve regulatory mechanisms for the release and utilization of glucose. Both the liver (25) and the intestinal mucosa (26) have been shown to be concerned with regulation of glucose release.

The demonstration of a steady state between blood glucose and expired CO_2 should allow a comparison to be made between the rates of oxidation of glucose by the two oxidative pathways. It has been shown that an increase in glucose oxidation by way of the EMP parallels the increase in expired CO_2 in the thyrotoxic rat. By contrast, glucose oxidation by way of the HMP is completely suppressed. The findings do not agree with those of other workers whose measurements were not made during a steady state. Spiro and Ball (13)

reported that the increased metabolic rate observed in the hyperthyroid rat reflects an approximately equal increase in the amounts of glucose metabolized by way of the EMP and the HMP. Glock *et al.* (11) reported greater participation of the EMP in hyperthyroid rat liver but no inhibition of the HMP. By contrast, in the steady state in the hypothyroid rat, reduction in glucose oxidation by way of both oxidative pathways has paralleled the reduction in total CO_2 production in the present trials.

Cahill *et al.* (27) have shown that the operation of the HMP is controlled by the level of oxidized TPN and Glock and McLean (12) demonstrated that TPN is preferentially present in its reduced form in the cell. Therefore, it would appear that the exclusive inhibition of the HMP oxidative pathway in the hyperthyroid animal must be due to the inhibition of processes which oxidize TPNH. Since DPN, in contrast to TPN, is preferentially present in its oxidized form in the normal cell, there exists an energetic potential favoring the hydrogenation of DPN at the expense of TPNH. However, an inhibitory action of thyroxine upon this pyridine transhydrogenase-catalyzed transfer of hydrogen has been demonstrated (7). It would appear then that the action of thyroxine involves the preferential maintenance of DPN in the reduced form. Since enzyme activities are not considered to be rate-limiting in most cases of biological oxidation (28), and since electron acceptor potentiality is tremendously greater than is ever utilized, even in hyperthyroidism, it would appear probable that the markedly hyperthyroid animal suffers from a deficiency of high-energy acceptors. DPN was found in an inhibited form in mitochondria lacking ADP (29). A deficiency of phosphorylated intermediates was suggested as the factor responsible for the reduced efficiency of energy utilization observed in the hyperthyroid rat since a beneficial effect of insulin was observed (17). Oxidative phosphorylation uncoupling agents have been shown to effect a preferential oxidation by way of the EMP (27).

The observed increase in glucose oxidation by way of the EMP has been shown to parallel the increase in rate of total oxidation in the hyperthyroid rat. The observed decreases in glucose oxidation by way of both oxidative pathways in the hypothyroid rat have been shown to parallel the observed reduction in rate of total oxidation. The results indicate that the effect of thyroxine is exerted on total oxidative activity and not specifically on glucose oxidation. The inhibitory effect of thyroxine on glucose oxidation by way of the HMP would appear to be a secondary effect of the increased demand for high-energy acceptors.

Acknowledgments

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References

1. F. L. Hoch and F. Lipmann. Proc. Natl. Acad. Sci. **40**, 909 (1954).
2. D. F. Tapley. J. Biol. Chem. **222**, 325 (1956).
3. G. F. Maley and H. A. Lardy. J. Biol. Chem. **215**, 377 (1955).

4. T. NECHELES and E. BENTLER. J. Clin. Invest. **38**, 788 (1959).
5. J. WOLFF and E. C. WOLFF. Biochem. et Biophys. Acta, **26**, 387 (1957).
6. J. G. WISWELL and M. G. BRAVERMAN. Endocrinology, **61**, 153 (1957).
7. A. M. STEIN, N. O. KAPLAN, and M. M. CIOTTI. J. Biol. Chem. **234**, 979 (1959).
8. N. O. KAPLAN, M. N. SWARTZ, M. E. FRECH, and M. M. CIOTTI. Proc. Natl. Acad. Sci. **42**, 481 (1956).
9. A. H. PHILLIPS and R. G. LANGDON. Biochim. et Biophys. Acta, **19**, 380 (1956).
10. A. S. FAIRHURST, J. C. ROBERTS, and R. E. SMITH. Am. J. Physiol. **197**, 370 (1959).
11. G. E. GLOCK, P. MCLEAN, and J. K. WHITEHEAD. Biochem. J. **63**, 520 (1956).
12. G. E. GLOCK and P. MCLEAN. Biochem. J. **61**, 390 (1955).
13. M. J. SPIRO and E. G. BALL. J. Biol. Chem. **231**, 31 (1958).
14. D. H. SOLOMON and J. T. DOWLING. Ann. Rev. Physiol. 615 (1960).
15. A. WHITE, P. HANDLER, E. L. SMITH, and DEW. STETTEN, JR. Principles of biochemistry. 2nd ed. McGraw-Hill Book Co. Inc., New York. 1959. p. 314.
16. B. MENDEL, A. KEMP, and D. K. MYERS. Biochem. J. **56**, 639 (1954).
17. D. S. DOW and C. E. ALLEN. Can. J. Biochem. and Physiol. **38**, 1471 (1960).
18. M. L. KARNOVSKY, J. M. FOSTER, L. I. GIDEZ, D. D. HAGERMAN, C. V. ROBINSON, A. K. SOLOMON, and C. A. VILLEE. Anal. Chem. **27**, 852 (1955).
19. L. I. GIDEZ. Ph.D. Thesis, Harvard University. 1953.
20. R. E. PETERSON. J. Clin. Invest. **37**, 736 (1958).
21. H. BROWN, E. ENGLERT, JR., and S. WALLACH. J. Clin. Endocrinol. **18**, 167 (1958).
22. H. HOLZER and I. WITT. Angew. Chem. **70**, 439 (1958).
23. E. H. STRISOWER and G. L. SEARLE. Brookhaven Symposia in Biol. **5**, 37 (1952).
24. G. A. REICHARD, B. FRIEDMANN, A. R. MAAS, and S. WEINHOUSE. J. Biol. Chem. **230**, 387 (1958).
25. G. F. CAHILL, J. ASHMORE, A. S. EARLE, and S. ZOTTU. Am. J. Physiol. **192**, 491 (1958).
26. W. C. SHOEMAKER, R. MAHLER, and J. ASHMORE. Metabolism, **8**, 494 (1959).
27. G. F. CAHILL, J. ASHMORE, and S. ZOTTU. J. Biol. Chem. **230**, 125 (1958).
28. E. G. KREBS and A. KORNBERG. Ergeb. Physiol. biol. Chem. u. exptl. Pharmakol. **49**, 212 (1957).
29. B. CHANCE and G. R. WILLIAMS. J. Biol. Chem. **217**, 409 (1955).

NOTES ON THE APPLICATION OF THE HERSCH GALVANIC OXYGEN CELL TO MEASUREMENT OF BLOOD OXYGEN CONTENT AND TENSION[1]

D. V. Bates and E. V. Harkness

Abstract

A number of galvanic oxygen cells have been built to the design of Hersch (1). Factors influencing the sensitivity of this type of cell to oxygen have been studied, and the potentialities of the cells in the analysis of small gas samples have been explored. With the cell used in the manner described, analysis of 105 samples of air of an average volume of 0.001 ml showed the standard deviation of the percentage of oxygen to be 0.9%. The mean oxygen percentage of these samples was correct at 20.8%. These cells were found to be suitable for the analysis of the oxygen contained in very small blood samples, and the method described probably offers the most precise technique there is for the determination of the oxygen saturation of 0.002 ml of blood. By bubble equilibration techniques, the cell may also be used to determine the oxygen tension of blood samples in the range 0–200 mm Hg.

In 1957, Hersch (1) discussed the design of a new type of oxygen-sensitive cell which was capable of analyzing the oxygen content of gases or fluids with considerable precision. Gray (2) described an application of this type of cell in the study of the oxygen tension of biological fluids, and in 1959 Deschner and Gray (3) extended these observations and described a new technique for the introduction of samples into the gas stream. In common with these authors, we feel that the Hersch cell may well be found generally useful in a number of biological problems, and for this reason have judged it worth while to present our experiences with the technique over a 4-year period. The theroetical considerations underlying the design of this type of cell have been fully discussed by Hersch and need not be recapitulated.

Technical Details

1. Apparatus

The sandwich cell described by Hersch has been used during the present investigations, and is illustrated in Fig. 1. It consists of a central strip of lead (15 cm × 1.2 cm × 2 mm) sandwiched between two 1-mm thick strips of porous polyvinyl chloride (porvic S), a material used for battery separators. These strips are themselves covered at the outer face by two strips of bright silver gauze, 24 mesh/cm and 80 mesh/cm respectively. The seven components are bundled together with silver wire, particular care being taken that the gauze does not touch the lead at any point. When this assembly has been constructed, it is submerged in 5 *N* potassium hydroxide in a large test tube. The air in the interstices of the gauze is then removed by allowing the gauze to remain in a

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vacuum for several hours until all bubbles have stopped emerging. After this, the sandwich is placed in its housing as shown in Fig. 1. It is important that it is then exposed immediately to an atmosphere of pure hydrogen, and we have noted some loss in sensitivity if there is much time delay between the impregnation process and the exposure of the cell to the atmosphere of pure hydrogen.

FIG. 1. The Hersch sandwich cell assembly shown in its housing. The cell itself is 15 cm long by 1.2 cm wide.

In Fig. 2, the Hersch cell is shown in the circuit which has been used in this study. The commercial hydrogen (H_2) is first brought from the tank through a demand regulator and then purified by passage through two Deoxo Catalytic

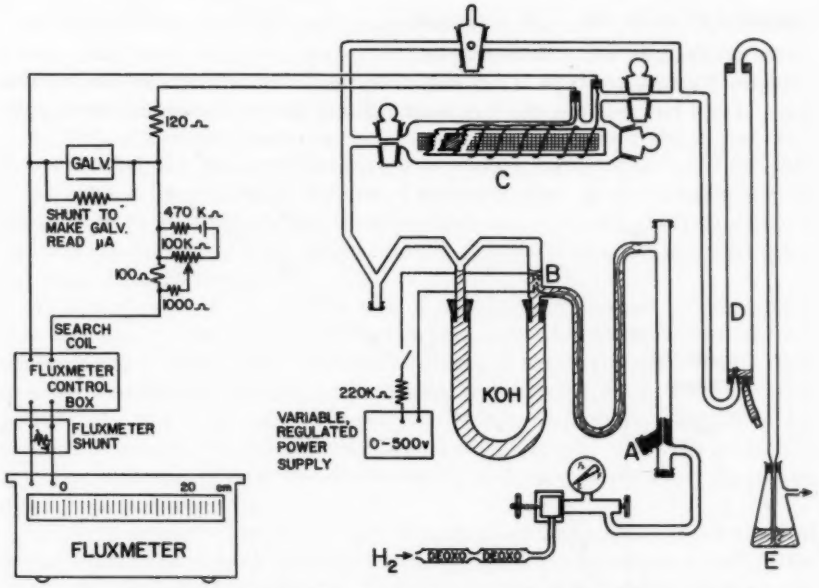

FIG. 2. The Hersch cell assembly (C) and circuit. Hydrogen is fed through two Deoxo purifiers and a sensitive needle valve into the circuit. Injections of unknown gases may be made at point A through a mercury seal held by a rubber cap. The electrolysis cell with electrodes at point B enable oxygen to be released into the circuit for calibration purposes. The cell assembly pictured in Fig. 1 is shown at C. D is a soap bubble flowmeter and E a wash bottle containing alcohol to dry the gas before it is released to the open air. The electrical connections of the cell are shown on the left-hand side of the diagram.

purifiers. Regulation of flow is accomplished by means of a sensitive needle valve. The hydrogen then passes through an injector cell (A, Fig. 2) which enables gas samples to be injected through a mercury seal into the hydrogen stream without leakage. The gas then passes into the electrolysis cell (B, Fig. 2), which contains 5 *N* potassium hydroxide. This cell is used in the manner described by Hersch to enable very small quantities of oxygen to be added to the gas stream electrolytically by calculation from Faraday's law. This gas is then allowed to pass over the Hersch cell (C) and then through a soap film flowmeter (D) to the atmosphere. The current put out by the cell is first monitored on a Rubicon galvanometer, which is shunted, and then through a fluxmeter control box to a sensitive large-scale fluxmeter (G.E. Model No. 9892910). After the cell has been run overnight in the pure hydrogen, the passage of a slug of oxygen liberated from the electrode or injected at point A will cause the galvanometer to show a rising and then a falling current. The fluxmeter will integrate this current, so that if it is started at zero, the light spot will go up to a convenient point and then remain stable. Readings are always taken at the end of 3 minutes. The use of a fluxmeter for this purpose was

suggested to us by Dr. L. H. Gray (2).

2. Calibration and Cell Characteristics

Figure 3 shows two typical calibration curves for the Hersch cell used in this way. It can be seen that the fluxmeter reading is at a convenient level when

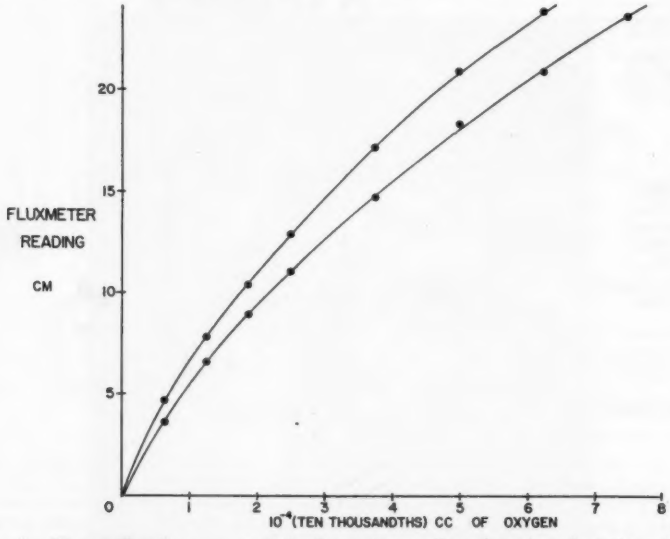

FIG. 3. Two calibration curves of the fluxmeter reading plotted against the volume of oxygen liberated by the electrolysis cell. Factors influencing the sensitivity of the cell assembly are discussed in the text.

about 0.0002 ml of oxygen is passed through the cell. The absolute sensitivity of any particular cell is dependent on a number of factors, of which we have found the following to be the most important.

(a) Recent oxygen passage.—As noted by Hersch, the continuous running of these cells in a completely oxygen-free atmosphere somewhat reduces their sensitivity to oxygen. This is observable when daily calibration work is being performed, since it will be noticed that the absolute sensitivity of the cell is invariably somewhat lower at the beginning of the week, after a week end during which no oxygen has passed through it. By the following day the cell sensitivity is back at its previous level, which it will hold for the rest of a week in which it is being used intermittently every day. In practice this has not been found to be a disadvantage since all readings are taken after two calibration oxygen runs have been made. The shift in sensitivity that occurs has not, we think, limited the accuracy of the cell to any significant degree. It is likely that the cell could be maintained at constant sensitivity by arranging for a small quantity of oxygen to be passed through it at regular intervals by using a time switch across the electrolysis cell.

(b) Moisture of cell.—It is very important that the cell is kept continuously moist. This is satisfactorily performed by the constant bubbling of hydrogen through the electrolysis cell. The hydrogen is left on day and night and the cell is satisfactorily moistened by this alone.

(c) Rate of hydrogen flow.—A constant percentage of the total oxygen that passes through the cell is reduced during its passage, but the absolute sensitivity of the cell will be affected by the rate of hydrogen flow. We have found 100 ml per minute to be a satisfactory rate for this, and it is important that this is checked intermittently with the soap bubble flowmeter to make sure that it has not been accidently changed.

(d) Age of cell.—There is some loss of sensitivity to oxygen in this type of cell, with age. In our experience this has been somewhat variable, but we have observed one cell that lost a significant degree of sensitivity after about three months; another cell has had no detectable loss of sensitivity over a 9-month period. The rate of decrease of sensitivity with age is probably affected by the amount of oxygen which has been passed across the cell. The cells are simple to make, however, and the loss of sensitivity with age has not proved a serious disadvantage in their use.

(e) Temperature.—There is a slight drop in sensitivity with a drop in ambient temperature, but if each unknown sample is balanced against a calibration point, this change is insignificant. The cell chamber is exposed to room air and it is not necessary for its temperature to be controlled.

Results

1. Analysis of Gas Samples

Samples of room air were analyzed for their oxygen content by injection of very small volumes of air into the apparatus at point A (see Fig. 2). The average volume of these samples was 0.001 ml of air, the gas being contained in a glass capillary syringe between two mercury columns. The gas volume was computed by measuring accurately the length of the sample in the capillary tube with a dissecting microscope. It was found convenient to attach a needle to one end of a capillary syringe for this work, since this needle could then be pushed through the rubber cap into the mercury at A (Fig. 2) and the air injected into the gas stream. The amount of oxygen the sample contained was computed from the fluxmeter reading, using a calibration graph similar to that shown in Fig. 3. The results of 105 such analyses were as follows:

number of samples	= 105,
average sample volume	= 0.001 ml of air,
average oxygen content	= 0.0002 ml of O_2,
mean O_2 % by analysis	= 20.8%,
range	= 19.7% to 22.3%,
standard deviation	= ±0.9%.

The sources of error in this type of analysis probably reside in the following two factors:

(*a*) the difficulty of measuring and handling very small volumes, and of insuring that only the measured sample is injected into the circuit for analysis;

(*b*) the variability of the fluxmeter. This instrument is sensitive to vibration and also to any marked temperature changes. Although it is reasonably satisfactory for routine use, it is probable that, if more attention were given to the very precise integration of the current output of the cell, even more accuracy would be achieved.

2. Analysis of Oxygen Content of Blood

One of the reasons for developing this circuit was to study its capabilities and potentialities in measuring the oxygen saturation of blood. In these estimates a 3-ml sample of blood was equilibrated in a tonometer with a known gas mixture. Two milliliters of the sample was then removed for O_2Hb determination using a Nahas cuvette and Beckman DU spectrophotometer (4). When the oxygen saturation of the blood sample had been determined with this cuvette, another sample was withdrawn for analysis in the Hersch cell. The sample volume varied between 0.002 ml and 0.010 ml of blood, and this was taken in a capillary syringe sandwiched between two mercury columns. At room temperature, the volume of the blood sample was measured by measuring the capillary column length with the dissecting microscope, and the sample was then injected into the circuit at A (Fig. 2). A very small quantity of acid ferricyanide, used in Van Slyke determinations, had previously been placed at the top of the mercury in A. The blood sample was then injected and the oxygen liberated from the blood produced a deflection on the fluxmeter in the usual way. We have noted that the oxygen released from the blood under these conditions comes off extremely quickly, and there is very little more delay in the fluxmeter traverse when blood is injected than when air is injected. The fluxmeter reaches its final reading about 140 seconds after it starts to move. The same blood was then equilibrated with room air and a further sample withdrawn for analysis in the Hersch cell so that the oxygen capacity of the blood might be calculated. Three determinations of oxygen content were made at a given saturation and oxygen capacity after room air equilibration, and the oxygen saturation of the sample was calculated by taking the mean of each of these three readings and dividing the oxygen content by the oxygen capacity. The results of these comparisons of oxygen saturation by these two methods are shown in Fig. 4. It will be seen that, over the whole range of saturation, satisfactory agreement was obtained between the two methods. It seems likely that the Hersch cell used in this way is capable of measuring the oxyhemoglobin saturation of 0.005 ml of samples of blood correctly to about 2% saturation when used in the manner described above.

3. Measurement of Oxygen Tension of Blood Samples

Freshly drawn blood was equilibrated with different oxygen concentrations in a tonometer rotated in a constant-temperature water bath at 37° C. Samples of blood were withdrawn from the tonometer using a 5-ml syringe ending in a

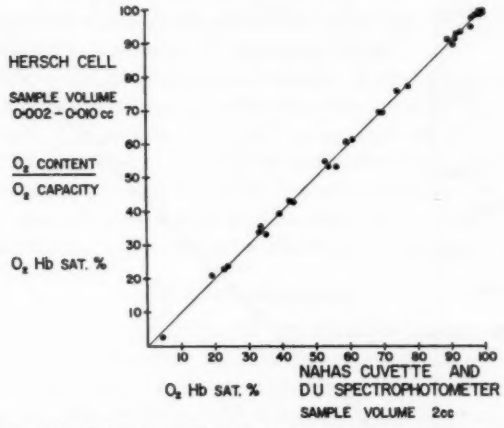

FIG. 4. On the *ordinate* is shown the oxyhemoglobin saturation of very small blood samples determined by analysis of oxygen content and capacity in the Hersch cell circuit. On the *abscissa* is shown the analysis of larger samples of the same blood using the Nahas cuvette (ref. 4). The agreement between these two methods with very different sample volumes is satisfactory over the whole range of saturations tested.

calibrated tube, and a small bubble of air was added to the syringe containing the withdrawn sample. This bubble was equilibrated with the blood by rotation in the water bath. The bubble was then trapped between blood and the shoulder of the syringe, and mercury was added to the cup at the top of the capillary tube. The bubble was now allowed to rise, so that it was trapped between mercury and blood. Using a tuberculin syringe fitted with a capillary tube, and ending with a hypodermic needle (25 gauge) sealed into the tube, the bubble was withdrawn from the first syringe into the second, where it was trapped between mercury seals. This bubble was returned to the water bath, where its volume was measured with the syringe under water, using a travelling dissecting microscope mounted outside the bath. The oxygen content of the bubble was then measured with the Hersch cell in the manner described above. Comparisons between the oxygen tension in the tonometer, measured by a Scholander apparatus, and the oxygen tension of the withdrawn blood sample measured as just described, are shown in Fig. 5. This shows generally satisfactory agreement between the two measurements though the Scholander gas equilibrating pO_2 is slightly higher than the Hersch cell blood pO_2 in a few instances. The mean difference between the estimates was +0.5 mm Hg pO_2 (Scholander minus Hersch). Individual differences ranged between +7.0 mm Hg pO_2 and −5.6 mm Hg, but in 23 of the 30 estimates between 0 and 200 mm Hg, the difference between the two determinations was less than 3 mm Hg. These differences are substantially greater than those reported by Deschner and Gray (3) for samples with a very low oxygen tension. The explanation of this difference probably lies in the following factors: the optimal method of

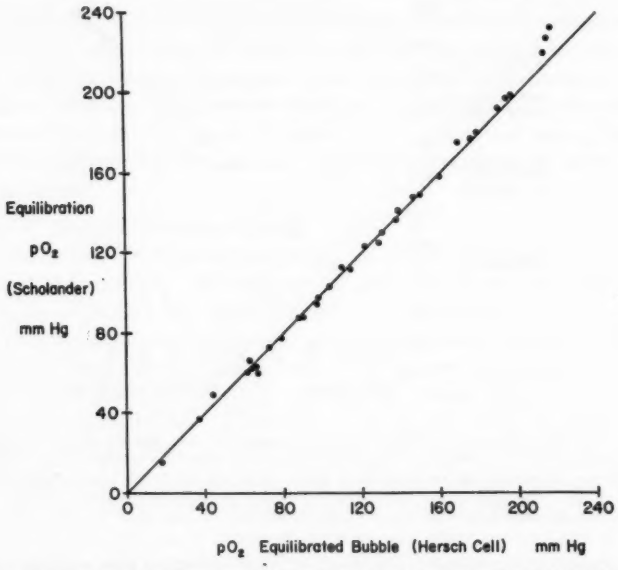

FIG. 5. Comparison between gas equilibration pO_2 determined by Scholander analysis and blood pO_2 determined by bubble equilibration and Hersch cell analysis.

bubble equilibration with fluid used by these authors instead of the standard bubble capillary technique available to us; the relatively greater precision of the Hersch cell at very low oxygen tensions; and, finally, possible technical differences in sampling handling or cell sensitivity.

Conclusion

The galvanic oxygen cell described by Hersch is undoubtedly capable of adequate stability and precision for the analysis of oxygen in very small gas or blood samples. The cell is unaffected by other common gases such as nitrogen, CO_2, CO, or inert gases of any kind, and its general characteristics make it suitable for bench use during research investigations. The extreme sensitivity of this type of cell to oxygen makes it ideal for oxygen analysis when only minute samples of gas or fluid are available for study. For this reason it should have a considerable field of application in biological investigations in which the samples available for analysis are of the order of a ten thousandth (0.0001 ml) of gas or fluid. The absolute accuracy of the method could probably be improved still further if extreme care were to be taken with the precise measurement of very small volumes, and if the variability of the fluxmeter could be overcome. A more elaborate integrating circuit with some form of recorder attached to it might well be capable of reducing the variation in the recorded cell output on standard calibrating analyses. However, even without such

refinements and used in the way described, it is clear that this type of cell could be usefully employed in a number of biological fields.

For the analysis of the hemoglobin saturation of blood samples of the order of 0.002 ml, this method probably offers the most precise technique that has yet been described. The cell can be used to measure oxygen tension, though the usefulness of the method is reduced by the known limitations of all bubble equilibration methods. However, unlike the contemporary oxygen electrode techniques, this method possesses the advantage that it does not necessitate individual calibration on each sample of blood to be analyzed.

Acknowledgments

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References

1. P. Hersch. Instrument Practice, **II**, 817, 937 (1957).
2. L. H. Gray. Progress in radiobiology. Vol. I. *Edited by* J. S. Mitchell, B. E. Holmes, and C. I. Smith. Oliver & Boyd, London. 1956. pp. 267–274.
3. E. E. Deschner and L. H. Gray. Radiation Research, **11**, 115 (1959).
4. G. G. Nahas. J. Appl. Physiol. **13**, 147 (1958).

SOME RESPIRATORY AND CARDIOVASCULAR EFFECTS OF GRADUAL SARIN POISONING IN THE RAT[1]

W. C. STEWART AND D. H. MCKAY

Abstract

Anesthetized rats were given sarin (isopropyl methylphosphonofluoridate) by slow intravenous infusion, while respiration, blood pressure, heart rate, and contractions of the gastrocnemius muscle in response to stimulation of the sciatic nerve were recorded.

When artificial ventilation was not carried out, breathing stopped after a toxic dose of sarin had been given, even though the blood pressure was above normal and neuromuscular conduction was not impaired. On the other hand, when artificial ventilation was provided after breathing stopped, the blood pressure and heart rate fell, and death was apparently caused by circulatory insufficiency.

Infusion of sarin caused slowing of the heart rate which was not prevented by previous section of the vagus nerves. Injection of atropine restored the heart rate to normal, and enabled the animals to withstand large doses of sarin as long as artificial ventilation was maintained.

It was concluded that sarin caused a cholinergic circulatory collapse which was the cause of death in rats maintained with artificial ventilation. This circulatory insufficiency was alleviated by large doses of atropine. Possible causes of the depression of circulation are discussed, and reasons are given for believing it to be due mainly to cholinergic diminution of cardiac output, caused by accumulation of acetylcholine in the heart.

Introduction

Sarin (isopropyl methylphosphonofluoridate), a highly toxic inhibitor of cholinesterase, has been found to cause death in the rat by central respiratory depression. When atropine was injected in large doses, the central depression was prevented, but respiration failed at a slightly greater dose of sarin, because of neuromuscular blockade (1). As might be expected, the lethal dosage for sarin is only slightly greater in rats injected with atropine than in unprotected controls (2).

It was thus apparent that sarin caused death in the rat by respiratory paralysis, either central or peripheral. It was therefore naturally of interest to investigate the possible toxic effects of sarin when asphyxia was prevented by artificial ventilation.

Methods

Adult male rats were anesthetized with intraperitoneal Dialurethane. Heparin sodium (100 units) was injected intramuscularly. A cannula was tied into the trachea for provision of artificial ventilation.

Freshly distilled sarin was diluted in saline to an appropriate concentration just before each experiment. The solution was given to the rats by slow intravenous infusion: for this purpose a polyethylene cannula was tied into the internal jugular vein, pointing toward the heart. A smaller polyethylene tube

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was passed through the cannula, so that the tip of the inner tube lay in the lumen of the vein near its junction with the subclavian vein. The inner tube was sealed to the cannula by an external polyethylene sleeve. The solution of sarin was then pumped through the inner tube into the blood stream of the rat. The pump was a micrometer microsyringe driven by a small electric motor.

In the experiments in which no artificial ventilation was given, a record of respiratory minute-volume was obtained. For this purpose, inlet and outlet valves were attached to the tracheal cannula. The air exhaled from the outlet valve was received in a tambour. After a fixed interval (usually 20 seconds), the tambour was automatically vented to the atmosphere through an electric hosecock. The resulting record was a series of vertical lines, of which the vertical excursion was approximately proportional to the respiratory minute-volume of the rat (see upper trace, Fig. 2).

In experiments in which artificial ventilation was given, oxygen was supplied to the tracheal cannula at two alternating pressure levels, i.e. atmospheric and 10 cm of water above atmospheric. The alternation of pressures took place at the rate of 1 cycle in 2 seconds. The alternation was controlled by a cycle timer and electric hosecocks. The arrangement was such that during the exhalation phase the tracheal cannula discharged air into a recording tambour initially at atmospheric pressure, thus providing a record of the artificial tidal air volumes. Because there was no valve between the tracheal cannula and the tambour, any spontaneous respiratory efforts made by the rat were also recorded, superimposed on the artificial volume changes (see upper trace, Fig. 3). During the inhalation phase of artificial ventilation the recording tambour was closed off from the trachea and vented to the atmosphere.

It was necessary to devise a method for recording the respiratory efforts of the rat: this was done by periodically stopping the artificial ventilation for a short time, while a record of the spontaneous respiration was taken. A second tambour connected directly to the tracheal cannula was used. Because the recording period was short compared with the intervening periods of artificial ventilation, accumulation of carbon dioxide during the interruption was slight. The necessary alterations in the electric and pneumatic connections were made at regular intervals by a cycle timer. Details of the arrangement are shown in Fig. 1.

Blood pressure was recorded from a cannula in the common carotid artery. The cannula was connected to a Statham pressure transducer by a short length of nylon tubing. A solution of heparin was pumped through the transducer dome and cannula to prevent the blood from clotting. The pressure was recorded by measuring the electrical output of the pressure transducer with a recording milliammeter (Fisher Recordall). The movements of the recording stylus were transmitted to the kymograph through a cord and system of pulleys.

Heart rate was recorded from the pulsations of the blood pressure as registered by the recording milliammeter. The oscillations of the recording stylus served to operate a mechanical friction clutch, which, in turn, opened and

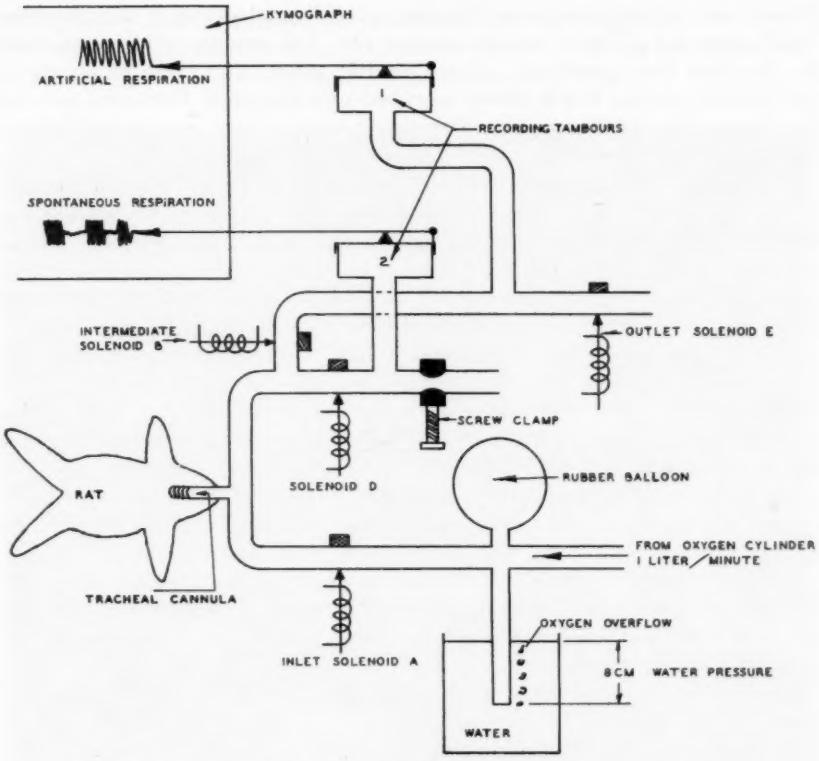

FIG. 1. Arrangement for providing and recording artificial ventilation in rats.

Oxygen at a constant pressure of 8 cm of water was provided on demand from the overflow system shown at the lower right. The electric solenoid hosecocks A, B, C, D, and E controlled the flow of oxygen to the recording tambours. These solenoids were themselves controlled by a cycle timer. The operating cycle of the system was as follows:

STEP NO.	TIME	ACTION
1	0	All solenoids close
2	1 second	Solenoid A opens, inflating lung; solenoid E opens, venting tambour 1
3	2 seconds	Solenoids A and E close; therefore all solenoids are closed
4	3 seconds	Solenoid B opens, connecting tracheal cannula to tambour 1, thus recording the artificial respiration
5	4 seconds	Solenoid B closes; this means all solenoids are closed as in step 1 and a cycle of events has been completed
6	From 4 seconds to 2 minutes	The above cycle is completed 30 times, providing and recording artificial respiration as many times
7	2 minutes	All solenoids are closed. E opens, thus interrupting the artificial ventilation; this means tambour 1 is removed from the circuit. Solenoid D opens; this means tambour 2 is cut into the circuit, and the animal, removed from artificia respiration, is permitted to respire spontaneously. This is recorded on tambour 1 for 30 seconds
8	2 minutes and 30 seconds	All solenoids close and the second cycle of events is completed. The first cycle, providing artificial respiration, begins as in step 1

closed a set of electric contacts. The contacts delivered a series of electric pulses which operated a Thorp impulse counter (3). The impulse counter was reset to the base line periodically by an electric timer. The cycle of events at the impulse counter was to record heart beats for 4 seconds, then reset and rest for 16 seconds, and then repeat. This gave a series of separate vertical lines on the record, each proportional in height to the heart rate.

Contractions of the gastrocnemius muscle in response to electrical stimulation of the sciatic nerve were also recorded. The left tendo Achillis was cut distal to a ligature: the ligature was led through a series of pulleys to a spring-loaded recording lever. Electrical stimuli were delivered through silver electrodes to the uncut sciatic nerve, which was exposed in the thigh. Two different kinds of stimuli were used: (*a*) single shocks (square waves, 6 volts, 10 milliseconds, 1 per second for 30 seconds), and (*b*) rapidly repeated shocks (the same, but 40 per second for 30 seconds). These two kinds of stimulation were applied alternately at intervals of 2.5 minutes. In this way the sciatic nerve was given first a series of single shocks, causing the gastrocnemius to respond with a series of single twitches for 30 seconds: then, after 2 minutes' rest, the nerve was given a 30-second burst of shocks at 40 per second, causing a tetanic contraction of the muscle. After 2 minutes' rest, single shocks were again applied, and so on.

Because the sciatic nerve was not cut above the stimulating electrode, the stimuli also caused afferent impulses in the nerve. These impulses elicited respiratory and vascular reflexes, whose effects could be observed in the records of respiration and blood pressure.

By the methods described it was possible to record both artificial and spontaneous respiration, as well as heart rate, blood pressure, and muscular contractions of two types simultaneoulsy.

Results

A series of 18 experiments was completed, in which sarin was given by slow intravenous infusion. In these experiments, five different sets of experimental conditions were used, as shown in Table I. The records obtained in four typical experiments are reproduced in Figs. 2 to 5.

TABLE I

Variations of experimental conditions in the series

Experimental conditions	No. of replications
(1) Infusion of sarin; no artificial ventilation; no atropine; vagi intact	4
(2) Infusion of sarin; with artificial ventilation; no atropine; vagi intact	5
(3) Infusion of sarin; with artificial ventilation; vagi cut; no atropine	2
(4) Infusion of sarin; no artificial ventilation; vagi cut; no atropine	3
(5) Infusion of sarin; with artificial ventilation; atropine sulphate, 10 mg/kg; i.v. vagi intact	4

The Effects of Slow Infusion of Sarin without Artificial Ventilation

When sarin was slowly infused into the rat, and no artificial ventilation was provided, the effects were typically those illustrated in Fig. 2.

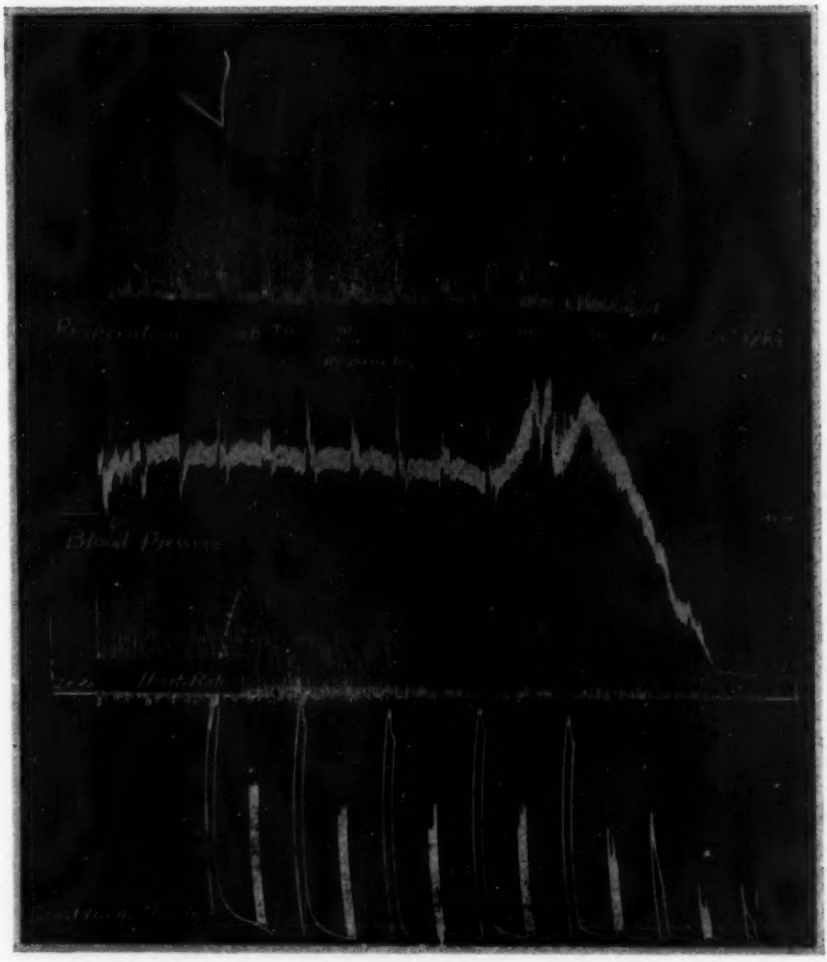

FIG. 2. The effects of slow infusion of sarin without artificial ventilation. Rat, 230 g. Infusion of sarin began at point marked GB.

Before the infusion began, the respiratory minute-volume was fairly constant, except when the sciatic nerve was stimulated (see upper trace, Fig. 2). At these times there were reflex increases in respiratory minute-volume due

to stimulation of afferent fibers. The reflex appeared to be elicited equally well by the two different types of stimuli applied.

After the infusion of sarin was started, there was no apparent effect on any of the recorded phenomena for a considerable time. When 35 to 40 μg/kg had been given, the first signs of poisoning suddenly appeared. These were a rapid rise in blood pressure, followed almost immediately by respiratory arrest, and severe bradycardia. The blood pressure then began to fall.

At this point some temporary recovery seemed to take place: weak, gasping, respiratory movements were accompanied by an increase in heart rate almost to the normal level, and by a resurgence of the blood pressure to a level considerably above the normal. The phase of partial recovery was followed by a second, more gradual, but final, deterioration.

The contractions of the gastrocnemius muscle were normal at the time of the first crisis, but became smaller soon after respiration ceased. When a tetanizing stimulus is applied to the nerve of a muscle in which partial neuromuscular blockade has been established by cholinesterase inhibition, the initial spike of the tetanic contraction is usually abnormally high, but the tension is not maintained as stimulation is continued. On the other hand, a series of single twitches often appears normal (4). In the experiments, exemplified by Fig. 1, this typical pattern of Wedensky inhibition was not seen: instead there was simply a decrease in the recorded heights of contraction, which may reasonably be attributed to anoxia of the muscle.

The reflex respiratory stimulation and changes in blood pressure caused by stimulation of afferent fibers in the sciatic nerve were no longer evident after the first signs of poisoning appeared.

The Effects of Slow Infusion of Sarin with Artificial Ventilation

The effects of slow intravenous infusion of sarin when the lungs were artificially ventilated are illustrated in Fig. 3.

In general, the effects of sarin on the respiration, heart rate, and contractions of the gastrocnemius were similar to those observed in the previous group of experiments in which no artificial ventilation was employed.

The blood pressure remained at a high normal level somewhat longer after respiratory depression and bradycardia had appeared than when artificial ventilation was not employed, but in the end the blood pressure fell rapidly to zero, and the heart stopped beating as before.*

*NOTE: It should be noted that the method of recording respiration used in Fig. 2 was different from that used in Figs. 3, 4, and 5. In Fig. 2, respiratory minute-volume was recorded, whereas in Figs. 3, 4, and 5 the record was of tidal volume, i.e. a recording of individual breaths. In Fig. 3, the small respiratory excursions recorded after 40 μg/kg of sarin had been infused were merely slow, irregular, and ineffective gasping movements, which appear somewhat magnified because of the slow speed of the recording paper.

It may also be seen that the excursions of the artificial ventilation record became somewhat smaller after spontaneous respiration ceased. The reason for this change was that because of the method of recording, while the rat was breathing, the volume changes due to the respiratory efforts were superimposed on the volume changes caused by the artificial ventilation: when the rat stopped breathing, the record gave a true representation of the passive volume changes taking place in the lungs.

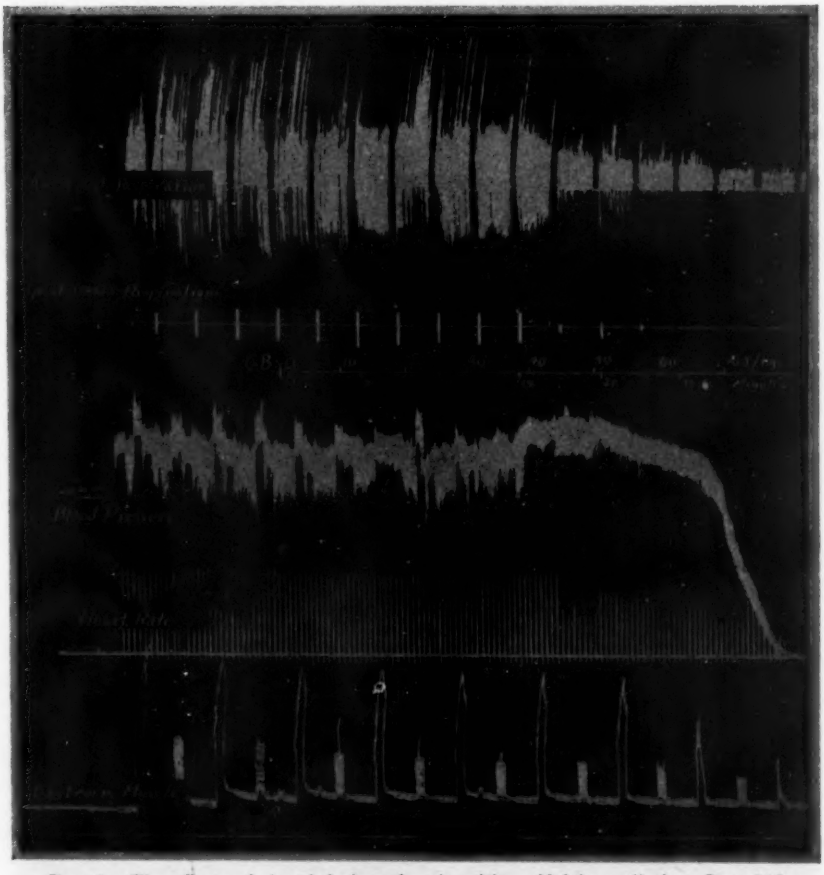

FIG. 3. The effects of slow infusion of sarin with artificial ventilation. Rat, 265 g. Sarin infusion began at point marked GB.

The Effects of Section of the Vagi on Sarin Bradycardia

The pronounced slowing of the heart rate observed during sarin poisoning in the rat could possibly have been the result of vagal nerve impulses. Cholinergic excitement in the vagus centers could cause a sudden increase in vagal cardioinhibitory impulses: or alternatively, cholinesterase inhibition at the vagal ganglionic synapses or at the postganglionic nerve endings in the heart might facilitate or magnify the effects of preganglionic impulses (5). In either case, cutting both vagus nerves in the neck would interrupt the preganglionic nerve impulses, and might be expected to prevent or modify the bradycardia caused by sarin. Accordingly, a number of experiments were done in which both vagi were cut about 20 minutes before the infusion of sarin: an

example is shown in Fig. 4.

Cutting the vagi did not prevent the bradycardia, nor did it change the pattern of development observed previously, in which there was a sudden slowing, followed by partial recovery, and then more gradual slowing to final arrest.

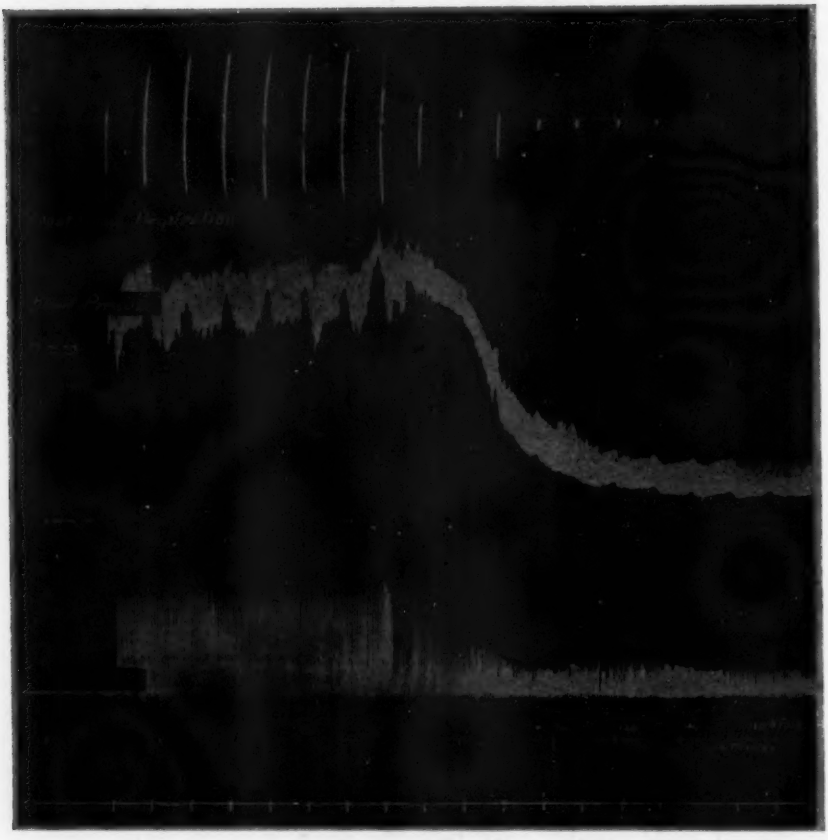

FIG. 4. The circulatory effects of sarin after section of the vagi. Rat, 270 g. Both vagus nerves were cut at the level of the thyroid cartilage, about 30 minutes before the beginning of the record. Infusion of sarin began at point marked GB.

The Effect of a Large Dose of Atropine

Figure 5 illustrates the results observed when a large dose of atropine sulphate (10 mg/kg) was injected intravenously during the cholinergic crisis.

In the particular experiment shown in Fig. 5, sarin was slowly infused intravenously in a rat maintained in artificial ventilation. After about 20 μg/kg had

FIG. 5. The effects of a large dose of atropine during sarin infusion. Rat, 272 g. Infusion of sarin began at the point marked GB. Atropine sulphate, 10 mg/kg, was injected intravenously as indicated on the record. Vagi intact. Infusion of sarin stopped at point marked GB off.

been given, there was the usual rise in blood pressure and fall in heart rate, accompanied by a partial depression of respiration. When the blood pressure began to fall, 10 mg/kg of atropine sulphate was injected intravenously: the result was an immediate increase in the heart rate to normal. The blood pressure, which was falling rapidly at the time of the injection, stabilized at a subnormal level. Spontaneous respiration did not return to normal, but instead continued in a depressed condition, gradually diminishing until 178 μg/kg of sarin had been infused; at that point the infusion of sarin was stopped. These reduced respiratory efforts were possible despite partial neuromuscular blockade, which was revealed by the inability of the gastrocnemius muscle to maintain a tetanic contraction. It seems likely that the partial neuromuscular block can be attributed to the combined action of sarin and atropine (6, 1).

Discussion

When sarin was gradually infused in the rat as described, no effects were observed until about two thirds of the usual intravenous lethal dose had accumulated. At this stage it may be presumed that cholinesterase inhibition had not progressed sufficiently to allow acetylcholine to accumulate. Scaife and Shuster (7) have shown that death took place in rats and guinea pigs when the brain acetylcholinesterase was reduced to about 25% of the normal value.

It seems clear that certain cholinesterase inhibitors cause depression of the respiratory center in most laboratory animals, rat, cat, dog, rabbit, and monkey (1, 8, 9, 10, 11, 12, 13). This interference with central control of the respiratory muscles is probably the cause of death from sarin poisoning in most species when respiration is not artificially aided (4, 9, 10, 11). Certainly in the rat there is little neuromuscular blockade at the time of respiratory arrest, providing the dose of sarin is not greatly in excess of the LD_{50} (1, 7).

In the rat, the arterial blood pressure is usually elevated above normal at the time of respiratory depression. This rise in blood pressure is due to peripheral vasoconstriction caused by cholinergic excitement of the vasomotor centers. Central vasomotor excitement in response to sarin appears to be a general phenomenon, observed in all the species studied so far (10, 11, 14). The rat differs from other species only in that the vasoconstriction is sufficient to raise the blood pressure above normal (15). In other species the vasoconstriction takes place, but is insufficient to maintain the blood pressure because of low cardiac output.

In the rat poisoned with sarin, and maintained on artificial ventilation, death is apparently due to circulatory insufficiency, indicated by the very slow heart rate and final fall in blood pressure. The present experiments provide no evidence to indicate in what proportion the final circulatory collapse is due to vasodilatation, and what to insufficient cardiac output. However, Fukuyama and Stewart (15) has shown that in the rat, the vasoconstriction persisted after the fall in blood pressure, indicating that low cardiac output was the predominant cause of the circulatory collapse. A similar conclusion has been

expressed by Holmstedt (4). Thus it seems that the vasomotor centers and the sympathetic ganglionic synapses which control vasomotor tone may be stimulated by cholinesterase inhibition, but are comparatively resistant to blockade from this source.

Cutting the vagus nerves did not prevent or modify the severe bradycardia observed during sarin poisoning in the rat: thus the bradycardia appears not to be of central origin, nor to be due to potentiation of the cardioinhibitory effects of vagal impulses. This observation agrees with similar experiments in other species (8, 16). A possible explanation of the bradycardia may be that cholinesterase inhibition allows acetylcholine to accumulate in the heart from sources within the heart itself: the source of the acetylcholine is not necessarily nerve cells. There is also a possibility that the cardiac slowing may be caused, in part at least, by the appearance of free acetylcholine in the blood stream (18).

When atropine was injected at the extreme dose of 10 mg/kg the rats were enabled to withstand comparatively large doses of sarin, but only if artificial ventilation was maintained. The cause of death in rats given atropine, but no artificial ventilation, is peripheral respiratory paralysis (1). The use of atropine renders the circulatory system very resistant to the effects of sarin. It is possible that very high doses of sarin might cause eventual circulatory collapse even in the atropinized animals, if a sufficient degree of ganglionic blockade were produced (18, 19).

References

1. W. C. STEWART. Can. J. Biochem. and Physiol. **37**, 651 (1959).
2. B. M. ASKEW. Brit. J. Pharmacol. **12**, 340 (1957).
3. R. H. THORP. Brit. J. Pharmacol. **3**, 271 (1948).
4. B. HOLMSTEDT. Pharmacol. Revs. **11**, 613 (1959).
5. H. WINTERBERG. Z. exptl. Pathol. **4**, 636 (1907).
6. C. TUM-SUDEN. J. Pharmacol. Exptl. Therap. **124**, 135 (1958).
7. J. F. SCAIFE and J. SHUSTER. Can. J. Biochem. and Physiol. **38**, 1087 (1960).
8. W. MODELL, S. KROP, P. HITCHCOCK, and W. F. RIKER, JR. J. Pharmacol. Exptl. Therap. **87**, 400 (1946).
9. C. A. DE CANDOLE, W. W. DOUGLAS, C. LOVATT-EVANS, R. HOLMES, K. E. V. SPENCER, R. W. TORRANCE, and K. M. WILSON. Brit. J. Pharmacol. **8**, 466 (1953).
10. R. V. BROWN. Brit. J. Pharmacol. **15**, 170 (1960).
11. H. POLET and A. F. SCHAEPDRYVER. Arch. intern. pharmacodynamie, **118**, 231 (1959).
12. B. METZ. Am. J. Physiol. **185**, 142 (1956).
13. R. V. BROWN, A. M. KUNKEL, L. M. SOMERS, and J. H. WILLIS. J. Pharmacol. Exptl. Therap. **120**, 276 (1957).
14. P. DIRNHUBER and H. CULLUMBINE. Brit. J. Pharmacol. **10**, 12 (1955).
15. G. S. FUKUYAMA and W. C. STEWART. Arch. intern. pharmacodynamie. To be published. 1961.
16. C. HEYMANS, A. POCHET, and H. VAN HOUTTE. Arch. intern. pharmacodynamie **104**, 293 (1955–1956).
17. K. E. ROTHSCHUH. Pflüger's Arch. ges. Physiol. **260**, 374 (1955).
18. W. C. STEWART. Brit. J. Pharmacol. **7**, 270 (1952).
19. K. KAMIJO and G. B. KOELLE. J. Pharmacol. Exptl. Therap. **105**, 349 (1952).
20. D. A. HOLADAY, K. KAMIJO, and G. B. KOELLE. J. Pharmacol. Exptl. Therap. **111**, 241 (1954).

THE PURIFICATION OF HAPTOGLOBIN[1]

G. E. CONNELL AND R. W. SHAW

Abstract

A method for preparing haptoglobin in highly purified form from human serum is described. The serum is acidified to pH 4.6 and deionized by gel filtration. Under these conditions haptoglobin is selectively adsorbed from serum by diethylaminoethyl-cellulose. The protein is eluted with a dilute solution of sodium chloride, and impurities are removed by fractionation with ammonium sulphate. A partial purification of serum cholinesterase can be obtained by a similar method.

Introduction

Haptoglobin is a protein found in the plasma of many mammalian species which forms specific complexes with hemoglobin (1). Human haptoglobin exhibits genetic polymorphism (2) and five distinct types of human haptoglobin have been described. The physiological significance of the protein has been investigated both in humans and in laboratory animals (3, 4, 5). Several methods for preparing haptoglobin in highly purified form have been described (6, 7, 8). The method of Connell and Smithies is based on selective adsorption of haptoglobin by an anion-exchange resin. The present method is based on the same principle, but certain modifications have been introduded which make the procedure simpler and applicable on a larger scale. A partial purification of serum cholinesterase by a similar method is also described.

Materials and Methods

Serum

Whole blood containing acid–citrate–dextrose solution is allowed to stand for several days in the cold until the cells are well settled. The plasma is aspirated and clotted as previously described (8). Individual samples of serum are classified according to haptoglobin type by starch gel electrophoresis (8) and are stored in polyethylene bottles at $-20°$.

Sephadex Column

Six hundred grams of Sephadex G-25 (Pharmacia, Uppsala, Sweden) is suspended in 3–4 liters 0.1 *M* NaCl and packed in a glass column 60 cm × 9 cm, provided with a coarse sintered disk. The column is washed with several liters of water saturated with toluene as a preservative. The flow rate should be about 20 ml/minute.

This column can be used repeatedly for desalting serum as described below. After six to eight batches of serum have been passed through it is advisable to remove the Sephadex from the column and wash it by stirring and settling in several 10-liter changes of distilled water.

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Contribution from the Department of Biochemistry, University of Toronto, Toronto, Ontario. This work was supported by a grant from the National Research Council of Canada.

DEAE-cellulose

Twenty-five grams of Selecta-Cel-DEAE-C (Brown and Co., Berlin, New Hampshire) is suspended in 1 liter 1 *N* NaOH, and stirred for 30 minutes. It is filtered by suction and the filter cake is washed with 2–3 liters water. It is suspended in water, titrated to pH 4.6 with 1 *N* HCl, and finally adjusted to a concentration of 25 mg/ml. (Used DEAE-cellulose can be regenerated by the same procedure.)

Preparative Procedure

Five hundred milliliters of serum is adjusted to pH 4.60 with 1 *M* acetic acid, using a pH meter standardized with 0.05 *M* potassium acid phthalate (pH 3.97 at 20°). The serum is layered carefully on top of the Sephadex column and filtered according to the procedure of Porath and Flodin (9). The desalted serum protein is collected in a volume of 600 ml. The specific conductance of this solution should not exceed 3×10^{-4} mho and pH should be 4.6 ± 0.10. The Sephadex column is regenerated by continued washing with toluene-saturated water.

Six hundred milliliters of desalted serum at pH 4.6 is treated with 5–20 g DEAE-cellulose in several successive treatments, using 0.5–2 g of the adsorbent in each treatment. The exact amount of DEAE-cellulose to be used is best determined by trial; it depends on several factors, for example, the capacity of the DEAE-cellulose, the efficiency of desalting, the concentration of haptoglobin in the serum, etc. The serum is stirred with the DEAE-cellulose for 15 minutes and is filtered by suction using a coarse filter paper (Eaton and Dikeman coarse creped paper is satisfactory). This step should take no more than a few seconds. The filter cake is washed with water, and then stirred up and packed carefully in a column provided with a coarse sintered disk. A column 2 cm in diameter is suitable for a 1-g portion of DEAE-cellulose. Elution with 0.2 *M* NaCl is begun and a faint-colored zone moves down the column with the sodium chloride front. The protein, chiefly haptoglobin, appears in the effluent practically at the sodium chloride front and can be collected in a few milliliters. The presence of protein in the effluent can be detected by measuring the optical density at 280 mμ.

The serum filtrate obtained from the first step is treated again with a fresh quantity of DEAE-cellulose of 0.5–2.0 g, and the procedure is repeated exactly as described above. It may be necessary to repeat this procedure 4–6 times to extract all the haptoglobin from the serum. The entire adsorption procedure should be completed within 1 to 2 hours.

The eluates from the successive treatments with DEAE-cellulose are analyzed for total protein by measuring the optical density at 280 mμ and for haptoglobin by the method of Connell and Smithies (8). Those eluates which are considered to be sufficiently rich in haptoglobin, for example those with a specific activity greater than 20×10^{-2} mg hemoglobin-binding capacity per optical density unit, are pooled.

The pooled preparation is diluted with water to an optical density of 10.0 at 280 mμ. Saturated ammonium sulphate solution is added with stirring to give a final concentration of 55% saturation. After 30 minutes the haptoglobin-rich precipitate is collected by centrifuging for 10 minutes at 12,000 g. The precipitate is washed by resuspending in 55% saturated ammonium sulphate solution and centrifuging again. The precipitate is dissolved in a minimum volume of water and dialyzed in the cold against several changes of distilled water. The dialyzed solution is freeze-dried.

Purification of Serum Cholinesterase (Pseudocholinesterase)

Serum and other materials are prepared as described for the preparation of haptoglobin except that the serum is not acidified prior to desalting. The serum in batches of 500 ml is passed through the Sephadex column and the desalted eluate is adjusted to pH 4.2 with 1 *N* acetic acid. The serum is then treated successively with several 0.5- to 2-g portions of DEAE-cellulose as described above and each portion is washed with water and eluted with 0.2 *M* NaCl. The eluates are assayed for cholinesterase by the spectrophotometric method of Kalow and Lindsay (10). The protein concentration is determined by measuring the optical density at 280 mμ. The fractions which are considered to be sufficiently rich in cholinesterase are combined.

Results

Haptoglobin Preparation

Table I summarizes the results of a haptoglobin preparation from 1400 ml of pooled serum of type 1-1. The serum was desalted on the Sephadex column in three batches. In this preparation six treatments with DEAE-cellulose were used and the greater part of the haptoglobin was obtained in the fourth, fifth, and sixth treatments. The eluates from these three treatments were combined and subjected to further fractionation with ammonium sulphate. The eluates from the first three treatments which were relatively poor in haptoglobin were discarded. An over-all purification of approximately 28-fold was obtained and the yield of purified material was 32% of the haptoglobin present in the original serum pool.

The eluates obtained in this preparation were analyzed by starch gel electrophoresis (11) and the results are presented in Fig. 1. The electrophoresis shows that the principal impurities in the preparation at this stage are proteins migrating faster than haptoglobin, chiefly albumin and an unidentified protein migrating between albumin and haptoglobin.

Figure 2 illustrates the further purification of haptoglobin by ammonium sulphate fractionation. The faster-moving impurities are almost entirely eliminated by this treatment. In longer electrophoretic runs ceruloplasmin can be detected as an impurity in the final preparations. It migrates slightly faster than type 1-1 haptoglobin and in amount it does not exceed 5% of the total protein judging by staining intensity. It is possible to separate ceruloplasmin

TABLE I

A typical preparation of haptoglobin

	Volume (ml)	Total protein spectrophotometric units (E_{280} mμ × vol.)‡	Hemoglobin binding capacity (mg)	Recovery of haptoglobin (%)	Specific activity, (hemoglobin cap./spectr. units) ×100
Serum	1,400	63,500	897	100	1.41
Desalted serum, pH 4.60	1,800	51,200	735	82	1.43
Eluate from 1st adsorption*	10	161	4.5	0.5	2.80
Eluate from 2nd adsorption*	10	213	12	1.3	5.63
Eluate from 3rd adsorption†	20	316	60.8	6.8	19.2
Eluate from 4th adsorption†	40	304	96.4	10.8	31.7
Eluate from 5th adsorption†	20	485	148	16.5	30.5
Eluate from 6th adsorption†	20	410	112	12.5	27.3
Residual serum	2,115	43,500	309	34.5	0.71
Ammonium sulphate fraction	20	718	284	31.7	39.6

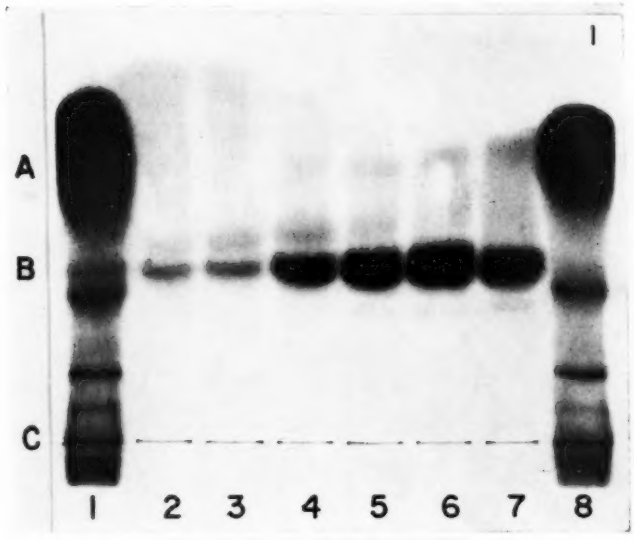
*1.4 g DEAE-cellulose was used. †2.8 g DEAE-cellulose was used. ‡Optical densities were determined on samples diluted 50× in *M*/50 NaOH.

TABLE II

Partial purification of serum cholinesterase

	Volume (ml)	Total protein spectrophotometric units (E_{280} mμ × vol.)	Esterase units*	Recovery of cholinesterase (%)	Specific activity, (esterase units/spectr. units) ×100
Serum	1,500	59,900	2,460	100	4.1
Desalted serum, pH 4.20	1,854	40,700	1,940	79	4.8
Eluate from 1st adsorption†	10	359	348	14	96.9
Eluate from 2nd adsorption	10	198	700	31	354
Eluate from 3rd adsorption	5	111	196	8	177
Residual serum	1,885	37,300	234	10	0.6

*One esterase unit is that amount of enzyme which will hydrolyze 1 ml benzoylcholine in 3 minutes at 26° C under the conditions specified by Kalow and Lindsay (10).
†1.5 g DEAE-cellulose was used in each adsorption step.



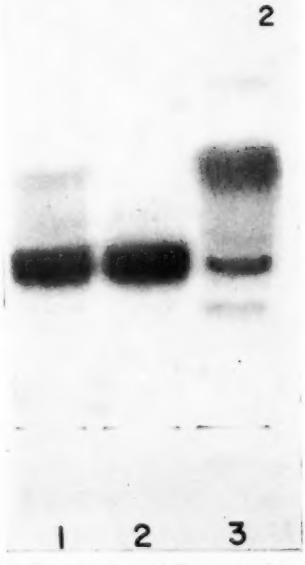


FIG. 1. Starch gel electrophoresis of partially purified haptoglobin after elution from DEAE-cellulose: A, albumin; B, haptoglobin; C, sample slats. 1, original serum; 2–7, eluates from successive batchwise treatments with DEAE-cellulose; 8, residual serum after treatment.

FIG. 2. Starch gel electrophoresis illustrating further purification of haptoglobin by ammonium sulphate fractionation: 1, pooled haptoglobin from DEAE-cellulose fractionation; 2, haptoglobin-rich fraction, precipitated at 55% saturation with ammonium sulphate; 3, impurities recovered at 80% saturation with ammonium sulphate.

and haptoglobin by chromatography on DEAE-cellulose (12). Ceruloplasmin can be readily separated from type 1-1 haptoglobin by further ammonium sulphate fractionation, but not from haptoglobin of the other genetic types (13).

Figure 3 illustrates the sedimentation behavior of the final type 1-1 preparation in the ultracentrifuge. The preparation appears to be nearly homogeneous with only a small amount of material (approximately 6% of the total area under the curve) sedimenting more slowly than the main component.

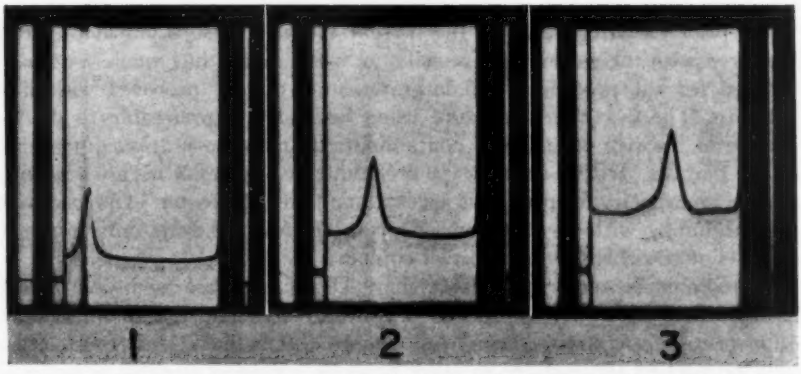


FIG. 3. Sedimentation patterns of purified haptoglobin preparation, approximately 1% protein in 0.18 *M* NaCl, 0.008 *M* NaH_2PO_4, 0.004 *M* Na_2HPO_4. Photographs were taken after 24 minutes, 56 minutes, and 96 minutes at 59,780 r.p.m. Temperature, 20.0° C.

Haptoglobin of all genetic types has been prepared successfully by this method from serum varying in amount from 10 ml to 5000 ml. The two most critical factors in the procedure are the pH and the ionic strength of the serum during the adsorption stage. The pH may vary from 4.4 to 4.8 without seriously affecting the yield or purity of the preparation. Below pH 4.4 haptoglobin is rapidly denatured as indicated by loss of ability to form a complex with hemoglobin. At pH 4.2 haptoglobin in whole serum is 80% denatured in 30 minutes. At pH values about 4.8 the selectivity of the adsorption decreases and therefore less pure preparations are obtained. If the ionic strength of the serum is not low enough, the efficiency of haptoglobin adsorption is much reduced. In some preparations the serum was desalted on Sephadex at neutrality and subsequently was acidified to pH 4.6 with acetic acid. These preparations gave adequate yields of haptoglobin but they were as a rule less pure than the standard preparations.

Cholinesterase Preparation

A typical preparation of cholinesterase starting with 1500 ml serum is summarized in Table II. In three adsorption and elution steps, 53% of the cholinesterase activity of the original serum was recovered and the specific activity was approximately 45 times higher than that of the starting material. The

second fraction alone contained 31% of the original amount of enzyme and the specific activity in this fraction was 86-fold higher than in the starting material. On starch gel electrophoresis the preparation appeared to be quite heterogeneous. Indeed, while several zones appeared in the gel on staining with amido black 10B none of these could be associated with the enzyme activity. Thus the present method represents only a partial purification of serum cholinesterase.

Discussion

The principal advantages of the present method of purifying haptoglobin compared with the method of Connell and Smithies are that much less time is required for the procedure and large amounts can be prepared with little difficulty. The desalting procedure using Sephadex is preferable to dialysis because of the risk of bacterial contamination and denaturation during prolonged dialysis. DEAE-cellulose is preferable to Dowex-2 as an adsorbent because its capacity for protein is several hundredfold greater. Thus preparations beginning with several liters of serum are feasible using only moderate amounts of adsorbent and no special equipment.

Haptoglobin is selectively adsorbed by DEAE-cellulose from solutions of low ionic strength at pH 4.6 because it is negatively charged while most other serum proteins are strongly positively charged. It is likely that this method could be applied successfully to other proteins, choosing suitable adsorbents, ionic strengths, and pH values. In the present investigation the method has been applied to cholinesterase and evidence has been obtained that it would be suitable for ceruloplasmin as well. Sanders *et al.* (14) have prepared ceruloplasmin from plasma by a similar procedure.

While large amounts of protein can be prepared by the batchwise method of adsorption, undoubtedly the resolving power of full-scale chromatography is lacking. Some of the selectivity of chromatography has been obtained, however, by using several small portions of adsorbent rather than one large portion. The proteins which have the greatest affinity for the adsorbent will be recovered from eluates from the first or second treatment while those with less affinity will be recovered from later treatments. By selecting those eluates richest in the desired protein a greater degree of purification can be attained than in a single adsorption step. This procedure is particularly advantageous when, as in the case of haptoglobin, the amount of the desired protein in the original solution is quite variable.

Acknowledgments

The authors are grateful to Miss V. Kask for valuable technical assistance and to Dr. W. Kalow and his staff for performing the serum cholinesterase determinations. Dr. John H. Crookston, Division of Haematology, Toronto General Hospital, and Dr. B. P. L. Moore and Dr. G. E. Stoker, Canadian Red Cross, very kindly arranged for the provision of the plasma used in this work.

References

1. M. POLONOVSKI and M. F. JAYLE. Bull. soc. chim. biol. **21**, 66 (1939).
2. O. SMITHIES and N. F. WALKER. Nature, **178**, 694 (1956).
3. C. B. LAURELL and M. NYMAN. Blood, **12**, 493 (1957).
4. W. LATHEM. J. Clin. Invest. **38**, 652 (1959).
5. R. K. MURRAY, G. E. CONNELL, and J. H. PERT. Blood, **17**, 45 (1961).
6. G. BOUSSIER. Compt. rend. acad. sci. Paris, **246**, 1769 (1958).
7. C. B. LAURELL. Clin. Chem. Acta, **4**, 479 (1959).
8. G. E. CONNELL and O. SMITHIES. Biochem. J. **72**, 115 (1959).
9. J. PORATH and P. FLODIN. Nature, **183**, 1657 (1959).
10. W. KALOW and H. A. LINDSAY. Can. J. Biochem. and Physiol. **33**, 568 (1955).
11. O. SMITHIES. Biochem. J. **71**, 585 (1959).
12. O. SMITHIES and G. E. CONNELL. Unpublished observations.
13. R. W. SHAW. M. A. Thesis, University of Toronto, Toronto, Ontario. 1961.
14. B. E. SANDERS, O. P. MILLER, and M. N. RICHARD. Arch. Biochem. Biophys. **84**, 60 (1959).

THE DISTRIBUTION OF POTASSIUM AND CAESIUM-137 IN THE CALF AND THE PIG[1]

R. M. GREEN, K. G. MCNEILL, AND G. A. ROBINSON

Abstract

Using a scintillation spectrometer, measurements have been made of the distribution of potassium and radioactive caesium in the bodies of the cow and the pig as a result of the ingestion of these materials. The results of these experiments, which are based on measurements of the radioactivity normally found in foodstuffs and in animals, agree with those of earlier experiments in that there is no great concentration of Cs^{137} apparent in any one organ. However, there is evidence for preferential concentration in the kidney and testes.

Introduction

The distribution of radioactive materials in the body has considerable bearing on the dose of radiation received by different organs. In particular we have been interested in the distribution of caesium-137 in the human body and its contribution to the possible somatic and genetic damage caused by fall-out. Experiments reported by Langham (1) suggest that Cs^{137} does not concentrate preferentially in the gonads of animals; however, concentration in other organs nearby could still increase the gonadal dose over the value calculated on the assumption that fall-out caesium is distributed uniformly throughout the muscle mass of the body. More generally, the finding of any large variation in concentration would make necessary the re-evaluation of current maximum permissible burdens.

There have been a number of studies of metabolism in animals after administration of radioactive caesium. For example, Hood and Comar (2) gave relatively large doses of Cs^{137} to pigs, cows, poultry, rats, and sheep, and subsequently they measured the distribution of the caesium in various organs of these animals. Hamilton (3) gave acute doses of various radioactive materials, including Cs^{137}, to rats and measured (i) the percentage accumulation in the principal organ and (ii) the rate of excretion. It is questionable whether the Cs^{137} distribution after an acute dose of 1 mc is necessarily the same as that which occurs after chronic doses of about 10^{-7} mc/day, and, therefore, whether these results are directly applicable to the fall-out problem, particularly when the variation in the biological half-lives for caesium in the rat and man shows that the metabolism of this element in the two species is different. Ballou and Thompson (4) compared the effects of acute and chronic doses in the rat, and found that the two methods of administration led to the same results. This tends to remove doubts about results obtained when acute doses are given, but even in these experiments with chronic doses, the daily dose is approximately

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Contribution from the Department of Physics, University of Toronto, Toronto, Ontario, and the Department of Physiological Sciences, Ontario Veterinary College, Guelph, Ontario.

one hundred times that obtained through fall-out. Moreover, the species distinction still exists.

The distribution of natural caesium in the "standard man", so far as it has been determined, is given in Table 7 of the "Recommendations of the International Commission on Radiological Protection, 1959" (5). For most organs, it is stated that the amount of caesium is less than the smallest measurable quantity.

Clearly it would be most correct to measure the Cs^{137} distribution in the various organs of a man who has had no contact with abnormal amounts of radioactivity, but this has not been possible. Experiments have therefore been carried out on the Cs^{137} distribution in the organs of cows and pigs of comparable weight fed on normal diets, and the results of these experiments are given here. Although the small amounts of radioactivity present make the detailed information available less than in the experiments previously quoted (1, 2, 3, 4), it was felt that the results would show up interspecies differences, if any, and help to confirm or deny the applicability of measurements of the effects of acute doses to the fall-out situation.

The scintillation spectrometry method used to measure the Cs^{137} activity also gave the distribution of potassium in the animals, as the naturally occurring K^{40} isotope emits gamma rays. A very complete picture of the potassium distribution in the standard man is given in Table 7 of the reference previously quoted (5). It is of interest to compare the results obtained in the present investigation with these figures for man.

Procedure

Thirteen Yorkshire pigs were fed on Hog Grower and water ad libitum for 6 weeks prior to slaughter. This was their only source of food and therefore the only source of the Cs^{137} subsequently found in the animals. At the time of slaughter their average weight was approximately 200 lb, and they would be classed as bacon hogs. The organs noted in the table were removed and placed in separate vessels designed to fit around a crystal detector. In all possible cases, parts from all pigs were included to average out effects of animal variation. The total weights used varied from 2 lb for lungs to 4.3 lb for female reproductive tracts. The variation in weight arose from the necessity of using identical geometries in every case. This density variation did give different absorption factors for each sample and individual corrections were applied for this effect. The pigs were slaughtered by exsanguination; however, no special effort was made to remove the blood from the organs after the pigs were bled as the object of the experiment was to find the distribution of Cs^{137} in the living animal. In order to preserve the specimens they were frozen.

Similar samples were obtained from five calves (approximately 290 lb body weight) which had been at pasture during the summer of 1960. They were fed mainly on hay, oats, and Calf Starter. Samples of these foods and the pig food were also taken.

The counting of the radioactivity of the specimens was carried out in the "steel room" of the University of Toronto (6). A long counting time was made necessary by the very low activity present, each sample generally requiring a counting time of 2 to 3 hours. A 5-in. diameter NaI crystal was used as detector. Such a crystal spectrometer can resolve gamma rays from different nuclei, thus making possible nuclide identification as well as intensity measurements.

The gamma-ray spectra in fact showed peaks due solely to K^{40} and Cs^{137}. Potassium-40, which decays with the emission of a 1.46-Mev gamma ray in 11% of disintegrations, is a naturally occurring isotope of potassium with an abundance of 0.0119%. Thus measurement of the K^{40} content gives the total amount of potassium present in a sample. Cs^{137} decays with emission of a 0.66-Mev gamma ray in 82% of disintegrations. By calibrating the system with a known amount of potassium chloride in the standard geometry, the absolute amounts of Cs^{137} and potassium in the samples were obtained.

Results

Table I, columns 2 and 3, shows the concentration of Cs^{137} in various organs of the pigs and the calves in units of micromicrocuries of Cs^{137} per gram of organ. Columns 4, 5, and 6 show the total potassium content, as a percentage of the organ weight, in the pig, calf, and man, respectively. Columns 7 and 8 give the caesium activity as a function of the total potassium content of the particular organ.

From the results of the table, which show a close similarity between man and the animals tested here, it may be deduced that the percentage by weight of potassium in the bodies of the cow and pig is approximately 0.2%, the value quoted for man (5).

Discussion

It is clear from the table that the percentages by weight of the potassium in the various organs of man are very similar to those in the cow and pig.

The values found for the total potassium content, expressed as a percentage by weight of the whole body, are in reasonable agreement with the determinations made using the biological half-lives and the rates of excretion of potassium in the cow and pig (7). From these latter measurements values for the total potassium content of 0.21% and 0.24% were obtained.

From a general survey of the caesium-to-potassium ratios for the two species and their foodstuffs, it is reasonable to conclude that the higher values for the pig reflect the higher caesium-to-potassium ratio in their food as compared with that in the cows' food. In this connection it may be noted that although the uniformity and density of the pig food enabled absolute measurements of both the potassium and the Cs^{137} content per gram of food to be made, the greater variety of the cow food and particularly the bulk of the hay prevented absolute measurements in this case, although relative measurements were possible.

TABLE I

Organ	Caesium activity* ($\mu\mu$c/g of organ)		Potassium content* (% of organ weight)			Caesium-to-potassium ratio ($\mu\mu$c Cs/g K)	
	Pig	Calf	Pig	Calf	Human†	Pig	Calf
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
Spleen	.111	.045	.35	.30	.35	30 ± 10%	15 ± 10%
Liver	.088	.019	.26	.27	.31	33	8
Kidney	.158	.075	.21	.17	.20	77	43
Small intestines	.079	.068	.17	.21	.15	44	32
Female reproductive tracts	.044	—	.17	—	.13	25	—
Brain	.062	.001	.25	.20	.33	24	5
Blood	.017	.0006	.09 ± 7%	.07 ± 7%	.01	15 ± 50%	12 ± 50%
Heart	.122	.058	.18	.19	.25	65	30
Pancreas	.067	.045	.20	.17	.28	31	26
Tongue	.098	.040	.18	.21	.28	54	18.5
Tenderloin	.134	.100	.26	.28	.28	52	36
Ham (thigh)	.177	.091	.29	.30		59	30
Body fat	—	—	0 ± .01	.03 ± .02		0	Indeterminate
Lung	.116	.055	.19	.20	.21	61	28
Ribs (bone and muscle)	.122	100	.20	.17	—	58	57.5
Boar's testes	.138	—	.20	—	.21	70	—
Feed	.116	—	.50	—	—	24	19

*Possible error on potassium measurements is ± 4%, and on caesium, ± 7%, except where otherwise noted.
†From reference 5.

Considering the relative activities of the various organs, it is seen that, in both the cow and the pig, the caesium-to-potassium ratio in the animal is greater than in the food, by a factor of about 2. On the widely accepted assumption that both potassium and caesium are totally absorbed from food into the body proper (5) this ratio should be reflected in the ratio of the biological half-lives of caesium and potassium in the two animals. The equilibrium half-lives for these elements have been estimated (7) and for both the pig and the cow $\tau_{Cs}/\tau_K = 1.4$. This ratio is in fair agreement with that quoted above, considering that τ_{Cs} is based on doubtful assumptions and on results on one cow and one pig.

On the question of the gonadal dose received from Cs^{137}, the table shows that there is some evidence that the Cs^{137} concentration is higher in the testes and kidneys than elsewhere in the body. The additional gonadal dose received from such a higher concentration in the kidneys will be small, but since approximately 60% of the dose received by an organ in the body from Cs^{137} is due to local beta radiation (8), it is clear that a high concentration in the testes could raise the dose to this tissue. However, it should be pointed out that the testes sample came from only one animal and therefore too much weight should not be given to this measurement. There is no evidence for any high concentration of caesium in the female reproductive organs.

The high concentration of Cs^{137} in the kidney suggests that either the kidney tissue, as such, preferentially retains caesium over potassium in a manner similar to that indicated for muscle by Relman (9), or the renal tubules reabsorb caesium in preference to potassium with a resultant low urinary clearance for caesium similar to that reported for the dog by Threefoot, Burch, and Ray (10). The low caesium concentration in the blood may be a consequence of the accumulation of this element by muscle and renal tissues.

This work, which is based on measurements of the radioactivity normally found in foodstuffs and in animals, confirms the results of the earlier experiments (1, 2, 3, 4) in that the Cs^{137} is mainly distributed uniformly throughout the body, and should help to remove doubts about the validity of earlier experiments carried out with large doses of radioactivity in specific animals.

Acknowledgments

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References

1. W. LANGHAM. Joint Committee on Atomic Energy of U. S. Congress. U. S. Government Printing Office, Washington. 1957. p. 741.
2. S. L. HOOD and C. L. COMAR. Arch. Biochem. Biophys. **45**, 423 (1953).

3. J. G. HAMILTON. Revs. Modern Phys. **20**, 718 (1948).
4. J. E. BALLOU and R. C. THOMPSON. Health Physics, **1**, 85 (1958).
5. RECOMMENDATIONS OF THE INTERNATIONAL COMMISSION ON RADIOLOGICAL PROTECTION, 1959. Health Physics, **3**, 148 (1960).
6. K. G. MCNEILL and R. M. GREEN. Can. J. Phys. **37**, 683 (1959).
7. G. A. ROBINSON, K. G. MCNEILL, R. M. GREEN, and H. C. ROWSELL. In preparation.
8. K. G. MCNEILL and H. E. JOHNS. J. Can. Assoc. Radiologists, **11**, March (1960).
9. A. S. RELMAN. Yale J. Biol. and Med. **29**, 248 (1956–57).
10. S. A. THREEFOOT, G. E. BURCH, and C. T. RAY. J. Lab. Clin. Med. **45**, 313 (1955).

STUDIES ON CLEARING FACTOR LIPASE (CFL) OF POSTHEPARIN PLASMA[1]

FRANK C. MONKHOUSE, JEAN STRACHAN, AND FLORENCE MCCLAIN

Abstract

A study has been made of factors effecting the postheparin plasma level of clearing factor lipase (CFL). A number of adsorbing agents and elution solutions were used to find optimal conditions for purification of CFL. Best results are obtained by adsorption on tricalcium phosphate and elution with 0.3% citrate. A study was made of CFL stability. Complete activity was lost in 7 days at 4° C, in 24 hours at 27° C, and in 6 hours at 37° C. Four species were studied. There was significantly less CFL activity in postheparin plasma of rats fasted 48 hours than in that of fed rats. Whole body irradiation of rats (600 r) results in a decrease in CFL activity of postheparin plasma but this decrease is less than in pair-fed controls. Thus, relative to food intake, irradiated animals have a higher than normal CFL activity. A crude extract of shellac when injected intravenously greatly increased CFL activity of postheparin plasma in the rat. This extract did not alter CFL activity of plasma in the absence of heparin and postshellac plasma added to postheparin plasma in vitro does not increase the activity of the latter. The effect on postheparin CFL activity increases with time after the injection of shellac. The activity is highest between 2 and 4 hours after injection.

When heparin is injected, an enzyme appears in the circulating blood. This enzyme has the ability to clear lipemic plasma and to hydrolyze triglycerides. The enzyme is seldom found in measurable amounts in normal plasma, nor is it activated in plasma in vitro on the addition of heparin. It has, however, been extracted from adipose and heart tissues (1), and has been demonstrated to be active in the epididymal fat pad of fed rats (2). Because of an apparent correlation between blood lipid levels and the development of atherosclerosis, a great deal of interest has recently been focused on this enzyme. Its physiological importance is at present unknown.

Methods of CFL Assay

Activity can be measured either by observing changes in light transmission, or by measuring the increase in FFA (free fatty acids) in a lipemic substrate following the addition of varying quantities of CFL.

Under ideal conditions the two methods of assay will give similar results. In many instances the results will not be similar. In most such instances it is well to rely on the release of FFA. Regardless of which method is used, it is absolutely essential to have a standard substrate system. Many workers (4, 6) have used commercial fat emulsions; others (5) have used chylomicrons from lymph. In our hands the most sensitive system has been dog chylomicrons added to high-spun oxalated dog plasma.

(*a*) *Chylomicrons*

The chylomicrons are prepared in the following way. A dog is fasted for

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Contribution from the Department of Physiology, University of Toronto, Toronto, Ontario.

18 hours and then is given a meal of meat supplemented with 5 ml of corn oil per kg of body weight. Two to three hours later the blood is collected in 1/10th its volume of 3.8% sodium citrate. After centrifugation to separate the cells, the lipemic plasma is transferred to plastic tubes and centrifuged at 10,000 r.p.m. for 1 hour at 4° C. The clear plasma is removed and the chylomicrons are then suspended in a small amount of pooled high-spun plasma and stored at 4° C. Chylomicrons so prepared and stored maintain their sensitivity to CFL for 1 week.

(*b*) *Pooled High-spun Plasma*

Pooled high-spun plasma is prepared by exsanguinating three to four large dogs into 1/10th volume of sodium oxalate. The red cells are removed by centrifuging at 2000 r.p.m. for 10 minutes and the resulting plasma is then centrifuged for 1 hour at 10,000 r.p.m. The plasma is placed in 50-ml plastic tubes, frozen, and stored at −20° C until used.

(*c*) *Standard Substrate System*

The changes in turbidity and FFA with a given preparation of clearing factor are dependent on the chylomicron concentration of the substrate. This is illustrated in Fig. 1. It can be seen that the optimal concentration in this

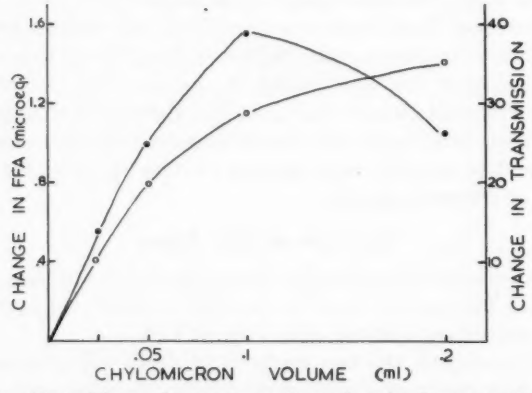

FIG. 1. The effect of increasing concentrations of chylomicrons on the activity of a constant amount of clearing factor lipase (CFL). The source of CFL is 0.2 ml of post-heparin rat plasma. ○ changes in FFA in microequivalents per ml; ● changes in transmission of light at 500 lambda.

instance is 0.1 ml of the concentrated chylomicron preparation in 2 ml of high-spun plasma. Since chylomicrons must be prepared at least once a week and will vary in concentration with each preparation, the amount added to the high-spun plasma for optimum conditions will vary. It can be seen from Fig. 2 that the turbidity of high-spun plasma at 500 lambda is proportional to the volume of chylomicrons added. It was found that the volume of chylomicrons which gives a transmission reading between 15 and 20 is optimal for CFL assay.

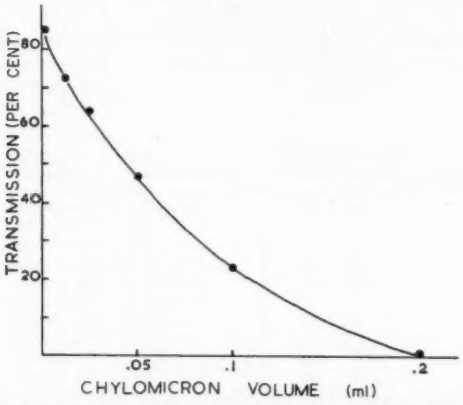

FIG. 2. The relationship between the percentage of transmission of light at 500 lambda and concentration of chylomicrons in mixed high-spun dog plasma.

In the following experiments CFL activity was assayed by adding 0.2 ml of postheparin plasma (or extract thereof) to 1.8 ml of the standard substrate. The mixture was incubated for 30 minutes at 37° C at a pH of 7.3. The FFA were measured by the method of Dole (3). The results are expressed as milliequivalents per liter or microequivalents per milliliter released per milliliter of postheparin plasma.

Some General Properties of CFL

CFL can be concentrated from postheparin plasma by cold ethanol precipitation (6) or by adsorption and elution (7). Table I presents a summary of results using a number of readily available adsorbing and eluting agents. It is clear that tricalcium phosphate for adsorption, and sodium citrate solution for elution, supply the only satisfactory combination. In subsequent experiments it was found that optimal conditions for elution were obtained using a volume of 0.3% sodium citrate equal to 20% of the original plasma volume. These results are in close agreement with those of Nikkila and his colleagues (7). The CFL can be concentrated from the eluate by dialysis and lyophilization. The results of a typical experiment on postheparin dog plasma are shown in Fig. 3. The yield in these experiments was low, the average being less than 20% of that calculated to be present in the original plasma. This is far short of the yield reported by Nikkila. All our final products contained much heparin and some prothrombin. Attempts to remove these by isoelectric precipitation resulted in large losses of activity. Dilution before adsorption, and treatment of the precipitate with oxalate before elution as recommended by Nikkila, did not significantly increase the yield.

During our attempts to purify CFL we became aware of the instability of the enzyme at room temperature. Since the stability seemed to vary with the

TABLE I

Adsorption of clearing factor from postheparin plasma

Adsorbing reagent	Concentration per ml plasma	Clearing activity		
		Supernatant	Eluate: Eluting reagent	Eluate: Activity
Ca_3PO_4	0.2 ml	Trace	Phosphate	− − −
		− − −	Tris buffer	− − −
		− − −	Na citrate	+++
$BaSO_4$	50 mg	+++	Na citrate	− − −
$BaCO_3$	50 mg	Trace	Na citrate	− − −
		Trace	Phosphate	− − −
$Al(OH)_3$	0.2 ml	+	Na citrate	+
		+	Phosphate	− − −
Fuller's earth	100 mg	++	Na citrate	− − −
Kaolin	100 mg	++	Na citrate	− − −

NOTE: − − − No activity. +++ Approximately 75%. ++ Approximately 50%. + Approximately 25% of the activity of original plasma.

Ca_3PO_4 suspension was prepared according to the method of Tiselius *et al.* (16). $Al(OH)_3$ was prepared as the C alpha product according to the method of Bertho and Grossman (17). $BaSO_4$ and $BaCO_3$, analytical grade, were obtained from J. T. Baker Chemical Co. Fuller's earth and kaolin were obtained from British Drug House Ltd.

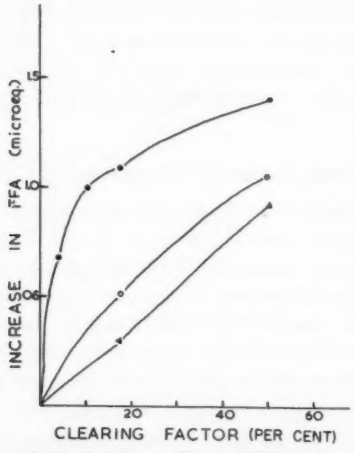

FIG. 3. Concentration of clearing factor lipase (CFL) from postheparin dog plasma. ▲ postheparin dog plasma; ○ citrate eluate; ● lyophilized citrate eluate. One milliliter of the lyophilized material represents 50 ml of the postheparin plasma. The abscissa refers to the percentage of the respective solutions.

species, we carried out a series of experiments on the stability of CFL of the rat, rabbit, guinea pig, and dog at different temperatures. Ten-minute postheparin plasma was used as a source of CFL and each experiment was carried out on pooled plasma from at least three animals. Typical results are shown in Fig. 4 for different species when postheparin plasma samples are kept at 4° C. At 27° C similar changes occur in less than 24 hours, and at 37° C in 5–6 hours.

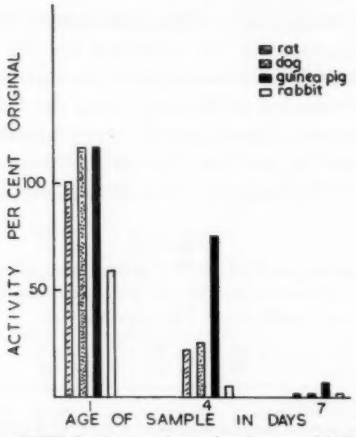

FIG. 4. The stability of CFL in the postheparin plasma of different species kept at 4° C.

The guinea pig CFL appears to be the most stable. There is, however, considerable variation in stability within the same species. The stability is directly related to the original activity; plasma CFL of high activity is more stable than that of low activity.

Factors Influencing the CFL Levels of Postheparin Plasma

As with other factors, the plasma level of CFL at any specific time is the result of the equilibrium between rate of entrance into the blood stream and rate of disappearance. As stated earlier, under normal circumstances plasma CFL is seldom measurable and this is probably because its rate of disappearance from the circulation is greater than its rate of entrance. The injection of heparin greatly increases the rate of entrance. With relatively small doses, the rise of CFL level and the duration of its presence in the plasma are proportional to the heparin dose. Beyond a certain dose, varying in different species (150 units/kg in dogs and rats), the level of CFL is not determined by the dose of heparin. Other factors which alter the CFL available can then be studied using the optimal dose of heparin to release it.

Recent studies (8, 9, 2) have shown that the CFL activity of incubated adipose tissue of rats is influenced by the nutritional state of the animal. Fasted rats show a significantly lower activity than the fed animals. Hollenberg (10) showed that the CFL activity of rat heart, in contrast to adipose tissue, was greater after fasting.

Many conclusions regarding the function of CFL have been based on plasma postheparin changes. Since fasting has different effects on different tissues, it was of interest to determine how fasting would influence the CFL level of postheparin plasma. Three separate experiments were carried out using a total of 11 rats. Each animal was injected with 100 units of heparin and 10 minutes

later a sample of blood was taken. The animals were then fasted for 48 hours and the procedure was repeated. As a control for the effect of the repeated heparin injection and sampling, five rats from the same age group were handled in a similar manner but were fed ad libitum during the 2-day interval. The CFL activities of the postheparin plasma samples were measured and the results are shown in Table II. It can be seen that there is significantly less CFL activity in postheparin plasma of fasted animals than in fed animals.

TABLE II

Effect of fasting on CFL level of postheparin plasma of rats

	First injection	Second injection	Difference
Treated (11)	1.91 $s = \pm 0.35$	1.44 $s = \pm 0.33$	-0.51 $s = \pm 0.15$
Controls (5)	1.81 $s = \pm 0.19$	1.86 $s = \pm 0.02$	± 0.05 $s = \pm 0.01$

NOTE: ( ) number of animals used.
Treated animals were fasted for 48 hours between heparin injections. Control animals were fed ad libitum during this time. All samples were taken 10 minutes after the intravenous injection of 100 units of heparin.
s refers to the standard deviation where

$$s = \sqrt{\left\{\Sigma(x^2) - \frac{(\Sigma x)^2}{N}\right\} \Big/ (N-1)}.$$

One of the observed changes following lethal doses of radiation in rabbits is the appearance of a visible lipemia (11). This lipemia can be decreased by the injection of heparin. In the rat, however, there is often a decrease in the opalescence of plasma during the first days after irradiation (12). Since heavy doses of radiation are followed by almost complete loss of appetite, it was of interest to study CFL activity in postheparin plasma of irradiated rats and compare the results with pair-fed controls. The results are shown in Fig. 5. Blood samples for measurement of CFL activity were taken 10 minutes after an intravenous injection of 100 units of heparin. Each point on the graph represents the mean value from eight rats. It is obvious that there is a significant decrease in the CFL activity of postheparin plasma of both the irradiated rats and their pair-fed controls. This decrease in CFL activity almost exactly mirrors the decrease in food intake. Nevertheless the irradiated animals do show significantly higher levels than their pair-fed controls over the first 5 days following treatment. Thus the postheparin plasma levels of CFL of the irradiated animals are higher than normal, relative to food intake.

During the course of some studies on the role of the liver in destruction of CFL, Higgins India ink was injected into rats. It was observed that the commercial ink caused a significant rise in total fatty acids in the blood (13). It was found that the lipemia was due to the shellac content of the India ink. When heparin was injected at the peak of the lipemia, the lactescence cleared and there was some reduction in total fatty acids. This was not sufficient to restore the blood level of fatty acids to normal. It was not due to the lack of CFL for, paradoxically, the plasma level of this enzyme was higher than in controls and persisted for a longer time at a high level. Dr. Milton Haines

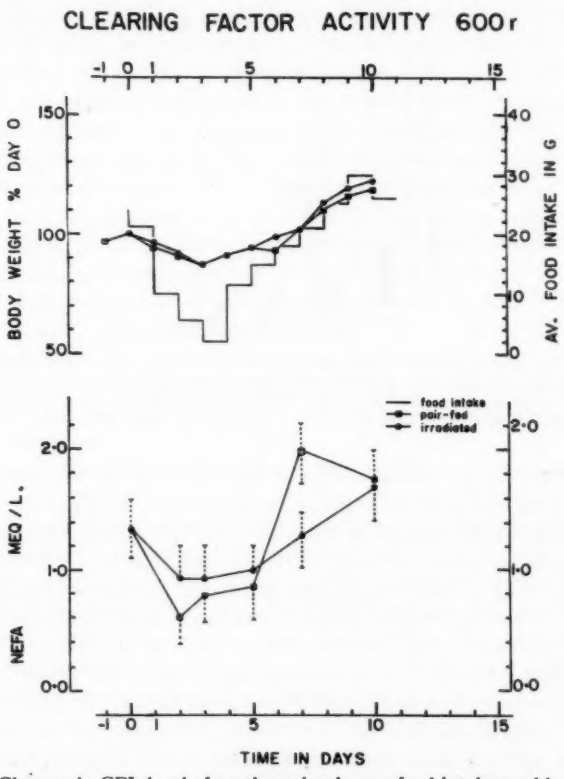

FIG. 5. Changes in CFL level of postheparin plasma, food intake, and body weight in rats following 600 r total body irradiation. Results from pair-fed controls are shown for comparison. Mean values and standard deviations are shown. Each point on the graph represents the mean value from eight rats.

working in the Best Institute has been able to partially purify the fraction from shellac which is responsible for both the lipemia and the increase in CFL activity. The crude shellac is shaken with an equal volume of chloroform, the mixture allowed to settle, and the supernatant discarded. This procedure is repeated three times following which the residue is taken up in a minimum volume of ethanol. The ethanol mixture is evaporated almost to dryness and stored in a tightly stoppered flask. For use, 10 mg of this tarry material is dissolved in 1 ml of weak ammonium hydroxide or 0.05 *N* sodium hydroxide. For the sake of brevity this material will hereafter be designated SF (shellac fraction).

In Fig. 6 a summary of the results of three separate experiments is shown. A total of 27 animals was used. Fifteen were injected with SF (0.6 ml per animal) and 1 hour later were given 100 units of heparin intravenously.

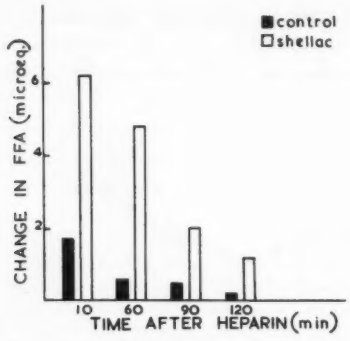

FIG. 6. The effect of a prior injection of shellac on the CFL activity of postheparin plasma from the rat. Heparin was injected 1 hour after the shellac. Each bar represents the mean value from seven animals. The 10-minute and 90-minute samples are from the same rats and the 60-minute and 120-minute samples are from the same rats.

Twelve rats were given saline followed by 100 units of heparin. Samples of plasma were taken at various intervals after the heparin injection. It is obvious there is a substantial increase in the CFL activity of the postheparin plasma in those animals receiving SF prior to the heparin injection. There are reasons to believe that this increase in activity is a result of real increase in CFL level in the blood. When postshellac plasma is added to postheparin plasma in vitro there is no significant increase in the CFL activity of the latter. It can be seen from Fig. 6 that CFL activity decreases rapidly after the initial rise. In these experiments the heparin was injected 1 hour after the SF. In other experiments, in which the heparin was injected at different times after SF, it was found that CFL activity of 10-minute postheparin plasma was greatest between 2 and 4 hours after the SF. Experiments are in progress on dogs in which an attempt is being made to concentrate the CFL activity from normal and SF-treated postheparin plasma. Neither the dog or rabbit, however, reacts to the SF in quite the same way as the rat.

Discussion

Clearing factor lipase (CFL) appears in the circulating blood almost instantaneously on the injection of heparin (14) and is blocked from entering the blood by protamine (15). Such evidence has given rise to the belief that the site of CFL release is the endothelial lining of the vessels and suggests that one function of CFL is to aid in the transportation of neutral lipids across the vessel wall. The experiments of Cherkes and Gordon (8) and Hollenberg (10) indicate that CFL is active in adipose tissue and heart muscle and may help to ensure an efficient distribution of the nutrient triglycerides during fasting. Nevertheless most opinions regarding the function of CFL and its involvement in the pathogenesis of atherosclerosis have been based on CFL changes in the blood.

The experiments reported here were designed to determine how changes,

which are known to alter fat metabolism at the cell level, alter the postheparin plasma levels of CFL. It was found that there was significantly lower CFL activity in postheparin plasma of fasted rats than in fed rats. When rats were given 600 r total body radiation there was a substantial decrease in caloric intake and an accompanying decrease in the CFL activity of postheparin plasma. This decrease was somewhat less than in the pair-fed controls. Thus treatment which would be expected to result in mobilization of fat from the fat depots and increased plasma levels of FFA (3, 16) resulted in a decrease in postheparin CFL. If the in vitro experiments of Cherkes and Gordon and Hollenberg are an indication of what occurs in vivo, then one must conclude that adipose tissue has a greater influence than muscle on the CFL activity of postheparin plasma. This would indicate that CFL is more important for the storage of fats than for their mobilization. Such results would also suggest that changes in CFL levels of postheparin plasma are more closely associated with changes in fat deposition in the fat stores than changes in transportation of lipid from the blood.

The foregoing discussion is based on the assumption that, with optimal doses of heparin, the CFL activity in the blood (or in the incubation mixture with in vitro experiments) is representative of the total amount of CFL available. Under ordinary physiological conditions there is no reason to doubt the validity of this assumption. The reason for the relatively large increase in CFL activity of postheparin plasma in the rat following the injection of shellac is at present obscure. The increased activity does appear to be a real increase in CFL level. Shellac extract does not increase the CFL level of plasma unless it is followed by an injection of heparin. Postshellac plasma added to postheparin plasma in vitro does not increase the CFL activity of the latter. Furthermore, the effect on postheparin CFL activity increases with time (up to 4 hours) after the injection of shellac. The shellac effect is not pronounced in the rabbit or dog.

Acknowledgments

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References

1. E. D. Korn and T. W. Quigley, Jr. Biochim et Biophys. Acta, **18**, 143 (1955).
2. D. S. Robinson. J. Lipid Research, **1**, 332 (1960).
3. V. P. Dole. J. Clin. Invest. **35**, 150 (1956).
4. E. D. Korn and T. W. Quigley, Jr. J. Biol. Chem. **226**, 833 (1957).
5. J. E. French, D. S. Robinson, and H. W. Florey. Quart. J. Exptl. Physiol. **38**, 101 (1953).
6. R. K. Brown, E. Boyle, and C. B. Anfinsen. J. Biol. Chem. **204**, 423 (1953).
7. E. A. Nikkila. Biochim et Biophys. Acta, **27**, 612 (1958).
8. A. Cherkes and R. S. Gordon, Jr. J. Lipid Research, **1**, 97 (1959).
9. C. H. Hollenberg. Federation Proc. **18**, 70 (1959).
10. C. H. Hollenberg. J. Clin. Invest. **39**, 1282 (1960).
11. R. L. Rosenthal. Science, **110**, 43 (1949).
12. D. G. Baker. Personal communications.

13. L. LICKLEY and F. C. MONKHOUSE. Proc. Soc. Exptl. Biol. Med. **103**, 777 (1960).
14. D. S. ROBINSON and J. E. FRENCH. Quart. J. Exptl. Physiol. **42**, 151 (1957).
15. F. C. MONKHOUSE, F. MCCLAIN, and D. G. BAKER. Proc. Soc. Exptl. Biol. Med. **102**, 50 (1959).
16. R. S. GORDON and A. CHERKES. J. Clin. Invest. **35**, 206 (1956).
17. A. TISELIUS, S. HJERTEN, and O. LEVIN. Archiv. Biochem. Biophys. **65**, 132 (1956).
18. A. BERTHO and W. GROSSMAN. Laboratory methods of biochemistry. MacMillan, London. 1938.

THE METABOLISM OF INOSITOL IN SALMON

III. THE BIOCHEMICAL REACTIONS OF 2-C^{14}-MYOINOSITOL IN COHO LIVER[1]

H. TSUYUKI AND D. R. IDLER

Abstract

The labelling of some of the intermediates of 2-C^{14}-myoinositol metabolism in acid extracts of coho salmon (*Oncorhynchus kisutch*) liver was investigated. Inositol monophosphate was found to be extremely heavily labelled. The specific activity of glucuronic acid was less than half that of the glucuronic acid moiety of uridine-5′-diphosphate glucuronic acid. Glucose, galactose, glycogen, glucose-1-phosphate, and glucose-6-phosphate contained very little radioactivity. The labelling data are discussed in relation to the known biochemical reactions of inositol in plants and animals.

Introduction

The distribution of radioactivities in the various tissues (1), and in the liver nucleotide fractions (2) of coho salmon following injection of 2-C^{14}-myoinositol, was previously reported. Of the liver nucleotides, the glucuronic acid moiety of UDPGA[2] was heavily labelled, while UDPH and UDPAH fractions showed little or no activity.

In the present communication the same acid-soluble liver extracts were explored further to determine the labelling pattern of some sugars, sugar phosphates, and other intermediates of inositol metabolism.

Methods

Injection of the Salmon[3] and the Preparation of Perchloric Acid Extracts

A 2340-g male coho was injected intraperitoneally with 3.98 mg (22 μc total activity) of 2-C^{14}-myoinositol and killed 7 hours later. An untreated 1860-g male coho was killed at approximately the same time and the livers, kidneys, guts, and hearts from the two fishes were combined as previously described (1). These samples were designated C^{14}-coho I.

In another experiment, a 4125-g male coho was injected intra-arterially with 3.65 mg of 2-C^{14}-myoinositol (20.3 μc total activity) in the dorsal aorta in three 1.0-ml doses at intervals of 2½ hours as described previously (1). Each dose was followed immediately by an intraperitoneal injection of 5.0 ml of

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[2]The following abbreviations are used in this manuscript: UDPGA, uridine-5′-diphosphate glucuronic acid; UDPH, uridine-5′-diphosphate hexoses; UDPAH, uridine-5′-diphosphate acetylhexoseamine; AMP, adenosine-5′-phosphate; CMP, cytidine-5′-phosphate; CDP-inositol, cytidine-5′-diphosphate inositol; G-1-P, glucose-1-phosphate; G-6-P, glucose-6-phosphate; GA-1-P, glucuronic acid-1-phosphate.

[3]We wish to thank Mr. Robert McLaren, Chief Biologist, Department of Fisheries, for making arrangements for us to obtain the salmon, which were captured immediately before their arrival on the Capilano River spawning grounds.

sterile 50% aqueous glucose solution. Four and one-half hours after the final set of injections, the fish was killed and the various tissues separated and stored. Samples from this fish were designated C^{14}-coho II.

The perchloric acid extracts of the liver were prepared by a slight modification (3) of the method of Hurlbert *et al.* (4).

Paper Chromatography

Chromatographic grade of Whatman Nos. 1 and 3 sheets, washed successively with ammonia, acetic acid, and methanol, were used (5). The chromatograms were subjected to descending development in Shandon[4] "chromatank" measuring 20 in. long by 8 in. wide by 20 in. high, usually at 20° C but also at 0° C when necessary to minimize decomposition. The solvent systems employed were as follows: IAW, isobutyric acid:ammonium hydroxide:water, 66:1:33; IAWE, same as solvent IAW but containing 10% ethanol; BPW, *n*-butanol:pyridine:water, 6:4:1; BAW, *n*-butanol:acetic acid:water, 4:1:5; aq. A, aqueous ammonia, pH 10.0; aq. B, water-saturated *n*-butanol; aq. BA, water-saturated *n*-butanol, containing ammonia in the bottom of the chromatographic tank.

When samples were eluted from chromatograms developed in IAW and IAWE, the ammonium ion was removed by treatment with Amberlite IR-120(H)[5] before reapplication to another chromatogram.

Location of Samples on Chromatograms

Ultraviolet-absorbing samples were located on a C-8016 Gordon Misco Ultraviolet Irradiator Blueprint[6] adapted to permit light from a clear G.E. germicidal lamp of maximum intensity at 253.7 mμ to pass through a Corning No. 9863 filter.[7] The hexoses were located by spraying with ammoniacal silver nitrate, and inositol, glucuronic acid, glucuronolactone as well as any other compound containing a vicinal glycol group by the periodate oxidation method of Yoda (6), except that the periodate concentration was reduced to 0.2% to increase the oxidation differential between the sample and the paper. Inositol could be detected at a level of 1 to 2 μg over an area of 2-cm diameter on Whatman No. 1 paper. Sugar phosphates and inositol phosphate were detected by the method of Wade and Morgan (7). To facilitate detection, the spots were viewed under ultraviolet light.

Analytical Methods

Hexoses were determined by the Folin and Malmros (8) method, glucuronic acid by a modification of the orcinol method of Meijbaum (9), and inositol by a slight modification (10) of the yeast assay method using *Saccharomyces carlsbergensis*.[8] When analyses were performed on samples eluted from paper

[4]Manufactured by Shandon Scientific Co. Ltd., 6 Cromwell Place, London, S.W. 7, England.
[5]Product of Rohm and Haas Co., Washington Square, Philadelphia 5, Penn., U.S.A.
[6]Manufactured by Microchemical Specialties Co., 1825 Eastshore Highway, Berkeley 10, Calif., U.S.A.
[7]This apparatus was modified by Mr. A. P. Ronald of this laboratory.
[8]The microbiological assay was carried out by Miss V. M. Chang of this laboratory.

chromatograms, a clear adjacent section of the chromatogram of equal size was eluted and an appropriate aliquot was used as a blank.

Results

Fraction A_3 for C^{14}-Coho II

This fraction, when chromatographed on Whatman No. 3 paper in solvent IAWE at 0° C, separated into four ultraviolet-absorbing bands. The largest and fastest-moving band corresponded to AMP in solvent systems IAW and IAWE and its base after formic acid hydrolysis was identified as adenine in solvents aq. A and aq. BA. The ultraviolet band moving just behind AMP moved like CMP in solvent IAW and after hydrolysis in formic acid the base was identified as cytosine in solvents aq. A and aq. BA. The remaining two faint and slower-moving ultraviolet bands were not investigated any further.

The radioactivity of fraction A_3 was concentrated in two areas of a chromatogram developed in solvent IAW. The major radioactivity band corresponded to glucuronic acid (4010 c.p.m.) and the minor band to glucuronolactone (196 c.p.m.). These two compounds accounted for virtually all the radioactivity in this fraction. Upon rechromatography in the same solvent system some of the glucuronic acid lactonized. The specific activity of the glucuronic acid determined by radioactivity measurements and by quantitative assay of the uronic acid on aliquots of the same sample was 1070 c.p.m. per μmole. There were 3.9 μmoles of glucuronic acid in this fraction.

Water Wash from Dowex-1-Formate Column

The first water-wash fraction referred to in the previous paper (2) and containing virtually all the radioactivity was freed of glycogen by the method of Stetten (11). The supernatant was concentrated and the residual glycogen precipitated. The combined supernatants from these operations were concentrated, dissolved in water, and deionized by passing through a mixed bed column of Amberlite IR-120(H) and IR-45 (OH). The eluant contained essentially all the radioactivity of the water wash indicating that little or no radioactivity was present in the glycogen.

One-quarter of the sample (2.5 ml) of deionized glycogen-free water wash was applied as a 7-in. streak across Whatman No. 3 paper and developed in solvent BAW for 40 hours along with glucose, galactose, and inositol as reference standards. The chromatogram was cut into strips horizontally along a line drawn across the reference standards spotted on either side of the unknown streak, it was eluted, and aliquots were counted. Any irregularities in the solvent front which occurred during a long-period chromatography would be detected in this manner. All of the radioactivity applied on the paper was recovered. The radioactivities were concentrated chiefly in the hexoses (135 c.p.m.) and the inositol (1238 c.p.m.) areas. Rechromatography of the hexose band in solvent BPW for 48 hours showed the presence of large amounts of glucose and a little galactose when sprayed with ammoniacal silver nitrate.

When another 2.5 ml of sample was treated in the same manner and the

hexoses eluted, 91 c.p.m. was present in the glucose band and 44 c.p.m. in the galactose band. The hexoses were determined quantitatively on an aliquot of the same sample used for radioactivity measurements. Expressed in terms of the entire sample, 5 μmoles of galactose of 35 c.p.m. per μmole specific activity and 78 μmoles of glucose of 4.7 c.p.m. per μmole specific activity were found.

Fraction A_5 of C^{14}-Coho II

One-tenth of fraction A_5 representing 5570 c.p.m. was streaked across Whatman No. 3 paper and chromatographed for 4 days at 0° C in solvent IAWE. Total separation of reference standards G-1-P, G-6-P, and GA-1-P was achieved. Inositol monophosphate, which moved 3.6 cm behind G-1-P was not completely separated from this sugar phosphate even after 4 days.

Of the total radioactivity of this fraction, 91% was recovered in the inositol monophosphate band, which, upon hydrolysis with 22% hydrochloric acid (10) followed by chromatography in solvent IAW for 2 days at 20° C, resulted in 90% recovery of the radioactivity in the free inositol area. Microbiological assay of the inositol indicated that 30 μg was present in fraction A_5. The inositol value must be considered as approximate since the amount assayed was near the lower limit of the microbiological assay. Quantitative assay of inositol monophosphate by total phosphate determination was not possible since a complete chromatographic separation between G-6-P and inositol monophosphate could not be achieved. On the basis of inositol assay, however, the specific activity of inositol monophosphate was 60,500 c.p.m. per μmole. Glucose-6-phosphate, G-1-P, and a trace of GA-1-P were detected in A_5 by chromatography.

Discussion

If the data for the free inositol content of male sockeye (10, 12) corrected for body weights are used to estimate approximately the content of inositol in the coho of comparable maturity used for intra-arterial injection (1), a figure of 199 mg is obtained. However, if it is assumed that the 3.65 mg of intra-arterially injected 2-C^{14}-myoinositol is present primarily in the kidney, liver, and alimentary tract after the relatively short duration of the experiment, a dilution with 23.7 mg of non-radioactive inositol is expected. The radioactive inositol, specific activity 622,000 c.p.m. per μmole, would be diluted to 83,100 c.p.m. per μmole and this is of the order of magnitude (60,500 c.p.m. per μmole) found for inositol monophosphate.

The specific activities of some of the intermediates of the metabolism of 2-C^{14}-myoinositol in the liver of coho salmon have been summarized in Table I. The specific activity of inositol monophosphate was found to be manyfold higher than any of the other components measured. Since its total radioactivity is only slightly less than one-half of the total radioactivity (2) of the acid extract, considerable inositol must be utilized in this way. Free inositol monophosphate, first isolated by Hawthorne and Hübscher (13) and Hübscher

TABLE I

Summary of specific activities

Compound	Radioactivity per μmole
Inositol monophosphate	60,500
Glucuronic acid from UDPGA	2,370*
Glucuronic acid (free)	1,070
Galactose	35
Glucose	4.7

*Value obtained from previous publication (2).

and Hawthorne (14) from guinea pig liver, could originate from direct phosphorylation of inositol, although Moscatelli (15) and Paulus and Kennedy (16) were unable to demonstrate an inositol "kinase" by in vitro incubation of inositol with tissue homogenates in the presence of ATP. It could also arise from the decomposition of CDP-inositol during acid extraction although Redman and Hokin (17) found this nucleotide inositol to survive the trichloracetic acid extraction procedure during its isolation from duck pancreas. The hydrolysis of inositol phosphatides could be another source. However, it is most likely that inositol is directly phosphorylated in vivo because of the high specific activity of its phosphorylated ester, when compared to the probable dilution of injected inositol. The ester could then be converted to CDP-inositol through reactions somewhat similar to the conversion of the C-1-phosphates of sugars and uronic acids to their corresponding UDP derivatives. CDP-inositol could be utilized further by conversion to inositol phosphatides by a series of reactions of the nature proposed by Agranoff *et al.* (18) and Paulus and Kennedy (16).

The experiments of Charalampous *et al.* with an enzyme system of rat kidney capable of cleaving the inositol ring point to glucuronic acid as being one of the first products of cleavage (9, 19, 20, 21). These authors were unable to find any intermediates in the conversion of inositol to glucuronic acid (21). In fish liver, however, the specific activity of the glucuronic acid moiety of UDPGA is more than twice that of free glucuronic acid. Although the specific activity data alone do not necessarily prove that UDPGA is a precursor of glucuronic acid in this conversion, they suggest that the eventual conversion of inositol to glucuronic acid may be accomplished either by a direct ring cleavage or possibly through a series of reactions leading first to UDPGA followed by its hydrolysis to the uronic acid. The manner in which such reactions occur is in the realm of speculation, since no data are yet available. The discovery of CDP-inositol (17) suggests that UDPGA could conceivably be formed from this source as well as from the presently known biochemical reactions outlined by Hassid *et al.* (22).

The low specific activities of the hexoses were not surprising since the hexoses are far removed from the chain of reactions involved in the eventual conversion of inositol to the hexoses. The very low labelling of glycogen is in agreement

with previous investigations where no measurable radioactivity was found in the liver glycogen isolated and purified from the water wash from Dowex-1-formate column of the acid extract of C^{14}-coho I.

References

1. H. TSUYUKI and D. R. IDLER. Can. J. Biochem. and Physiol. **38**, 1173 (1960).
2. H. TSUYUKI and D. R. IDLER. Can. J. Biochem. and Physiol. **38**, 1177 (1960).
3. H. TSUYUKI, V. M. CHANG, and D. R. IDLER. Can. J. Biochem. and Physiol. **36**, 465 (1958).
4. R. B. HURLBERT, H. SCHMITZ, A. F. BRUMM, and V. R. POTTER. J. Biol. Chem. **209**, 23 (1954).
5. D. R. IDLER, A. P. RONALD, and P. J. SCHMIDT. Can. J. Biochem. and Physiol. **37**, 1227 (1959).
6. A. YODA. J. Chem. Soc. Japan, **73**, 18 (1952).
7. H. WADE and D. MORGAN. Nature, **171**, 530 (1953).
8. O. FOLIN and H. MALMROS. J. Biol. Chem. **83**, 115 (1929).
9. F. C. CHARALAMPOUS and C. LYRAS. J. Biol. Chem. **228**, 1 (1957).
10. V. M. CHANG, H. TSUYUKI, and D. R. IDLER. J. Fisheries Research Board Can. **17**, 565 (1960).
11. D. STETTEN and G. E. BOXER. J. Biol. Chem. **155**, 231 (1944).
12. D. R. IDLER and H. TSUYUKI. Can. J. Biochem. and Physiol. **36**, 783 (1958).
13. J. N. HAWTHORNE and G. HÜBSCHER. Biochem. J. **64**, 53 p. (1956).
14. G. HÜBSCHER and J. N. HAWTHORNE. Biochem. J. **67**, 523 (1957).
15. E. A. MOSCATELLI. Biochemical studies on myoinositol. Ph.D. Thesis, University of Illinois, Springfield, Illinois. 1958.
16. H. PAULUS and E. P. KENNEDY. J. Biol. Chem. **235**, 1303 (1960).
17. C. M. REDMAN and L. E. HOKIN. Federation Proc. **19**, 309 (1960).
18. B. W. AGRANOFF, R. M. BRADLEY, and R. O. BRADY. J. Biol. Chem. **233**, 1077 (1958).
19. F. C. CHARALAMPOUS, S. BUMILLER, and S. GRAHAM. J. Am. Chem. Soc. **80**, 2022 (1958).
20. F. C. CHARALAMPOUS. J. Biol. Chem. **234**, 220 (1959).
21. F. C. CHARALAMPOUS. J. Biol. Chem. **235**, 1286 (1960).
22. W. Z. HASSID, E. F. NEUFELD, and D. S. FEINGOLD. Proc. Natl. Acad. Sci. **45**, 905 (1959).

DEOXYCYTIDYLATE DEAMINASE LEVELS IN REGENERATING RAT LIVER[1]

D. K. MYERS, C. ANNE HEMPHILL, AND CONSTANCE M. TOWNSEND

Abstract

Deoxycytidylate deaminase activity and net synthesis of deoxyribonucleic acid (DNA) in vivo were found to increase at approximately the same time during the early stages of liver regeneration. However, deaminase activity in the regenerating liver remained at a high level for 1 day after DNA synthesis had slowed down again during the later stages of regeneration. The increase in deaminase activity was restricted as a result of exposure to 600 r X radiation during early regeneration, but this effect only became evident 11–16 hours after the irradiation. Irradiation on the second day after partial hepatectomy, when deaminase levels in control regenerating livers were relatively constant, failed to affect the deaminase activity immediately but did produce a 40–50% decrease in activity 11–16 hours later. Other antimitotic agents, e.g., colchicine, had little effect on deaminase activity.

Introduction

The present investigation was concerned with the changes in activity of deoxycytidylate deaminase during liver regeneration and with the effects of X irradiation on this activity. Our interest in this enzyme arose from its association with the biosynthesis of DNA. The metabolic pathway leading from deoxycytidylic acid via thymidylic acid to DNA involves deoxycytidylate deaminase together with at least three other enzymes (thymidylate synthetase, thymidylate kinase, and DNA polymerase). The activities of all four enzymes rise from a low level in normal liver to much higher levels in regenerating liver (1–4). Although the changes in deaminase activity have not been examined in detail previously, the increase in the activities of the two enzymes involved in the incorporation of thymidylate into DNA (2) seems to parallel approximately the well-known increase in rate of DNA synthesis in vivo. These increases are both delayed following irradiation of the liver (5, 6). Since deoxycytidylate deaminase catalyzes an earlier step in this metabolic pathway, it seemed of interest to study the changes in activity of this deaminase during regeneration of liver in control and irradiated rats.

Methods

Deaminase Assay

The deamination of deoxycytidylic acid (dCMP) to deoxyuridylic acid and ammonia by tissue preparations has been studied by Maley and Maley (4) and by Scarano (7). The assay method described by Maley and Maley (4) was modified as follows in order to increase its sensitivity and reliability. Each gram of liver was homogenized with 2 ml of an ice-cold solution containing

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0.1 *M* KCl, 0.03 *M* Tris buffer (tris(hydroxymethyl)aminomethane), and 0.02 *M* EDTA (ethylenediaminetetraacetic acid); the final pH of the homogenate was 7.3–7.4. At zero time, 0.6 ml of the ice-cold homogenate was mixed rapidly with 0.4 ml of substrate solution and the mixture was incubated in a water bath at 37° C. The final composition of the reaction mixture was 0.005 *M* dCMP, 0.04 *M* KCl, 0.008 *M* EDTA, 0.01 *M* NaF, and 0.15 *M* Tris buffer at pH 8.0. Immediately after mixing this suspension, duplicate samples of 0.15 ml were added to 10 ml of cold 5% trichloroacetic acid; further samples were removed from the reaction mixture after incubation for 30 and 60 minutes at 37°. After centrifuging of the trichloroacetic acid suspension, the concentration of cytidine derivatives in the acid supernatant was estimated from the difference in optical densities at 290 and 300 mμ as measured with a Beckman DU spectrophotometer. Tissue controls, with dCMP omitted from the reaction mixture, exhibited little change in the difference readings during incubation under the above conditions. In a few experiments, dCMP was compared with 5-methyldeoxycytidylic acid (Me-dCMP) as substrate (Table I); in this case, the concentrations of both substrates were estimated from differences in the optical densities at 295 and 310 mμ.

TABLE I

Comparison of deaminase activities with dCMP and Me-dCMP as substrates

Tissue preparation	Deaminase activity (μmoles/g/hour)	
	dCMP	Me-dCMP
Regenerating liver	5.8	7.1
Supernatant from regenerating liver	5.6	7.0
Liver	2.0	2.5
Cervical lymph nodes	2.6	3.3
Spleen	3.3	4.3
Thymus	25.0	31.0
Oviduct	12.0	15.0
Testis	6.8	8.8
Kidney	(<0.4)	(<0.4)

NOTE: Regenerating liver was obtained from 10-week-old female rats 2 days after partial hepatectomy; the supernatant was prepared by centrifugation of this homogenate at 35,000 *g* for 30 minutes at 0° C. All other tissues were obtained from 5-week-old male and female rats.

The present assay was not affected by the addition of 0.01 *M* adenosine triphosphate to the reaction mixture in order to minimize the loss of substrate through enzymic dephosphorylation. No evidence for naturally occurring inhibitors or activators of the deaminase could be obtained by mixing homogenates from tissues with low and high enzyme activities. When homogenates of normal liver were added to homogenates either of regenerating liver or of thymus, the deaminase activities of the mixtures were equal to the sum of the activities of each homogenate.

Preliminary experiments with thymus homogenates, which contain a high deaminase activity (4), showed that the decrease in dCMP concentration during the assay was approximately proportional both to the time of incubation and to the concentration of enzyme, up to the point where 20% of the initial dCMP had been deaminated. Further deamination of the dCMP progressed more slowly, presumably due to the inhibitory effect of the deoxyuridylic acid produced in the reaction (4). All subsequent assays were therefore limited to less than 20–25% deamination of the initial substrate.

Animals

Treatment of the experimental animals followed that used in previous experiments on DNA synthesis and cell division (8, 9) as closely as possible. Female animals, weighing approximately 180 g, were selected from a laboratory stock of hooded rats when they were 10 weeks old. These animals were partially hepatectomized under ether anaesthesia by a standard surgical method to remove $68.4 \pm 1.5\%$ of the liver. After partial hepatectomy, each rat was kept in an individual cage in a ventilated room which was held at 24–27° C and artificially lighted for 12 hours each day from 6 a.m. to 6 p.m. (E.S.T.). Unless otherwise stated, all animals were allowed food and water ad libitum. Animals were killed for enzyme assays by decapitation under ether anaesthesia; a portion of the right liver lobe was removed immediately, weighed, and homogenized as described above. Subsequently the remaining portion of the liver was also removed from the animal and weighed in order to obtain total liver weight.

During X irradiation, unanaesthetized animals were restrained in a rectangular plastic chamber (8). The major portion of the animal was protected by lead shielding and the liver region was exposed through a 3.6-cm hole in the shielding to X rays from a 300-kv machine operated at 10 ma (inherent filtration equivalent to 5 mm Al). The dose rate, measured in air inside the plastic chamber at a distance of 20 cm from the faceplate, was 300 r/minute. Sham-irradiated animals were treated at the same time and in the same way as irradiated animals, except for the actual irradiation.

Results

Deamination of Deoxycytidylate (dCMP) and 5-Methyldeoxycytidylate (Me-dCMP)

The metabolic pathway from dCMP to thymidylate involves a deamination and a methylation of the pyrimidine ring (4). Although the sequence of these two steps is not certain, both dCMP and Me-dCMP can be deaminated by tissue preparations (4, 7). It was of interest, therefore, to determine whether both substrates are deaminated by a single enzyme in rat liver. This suggestion was confirmed by three findings: The ratio of activities towards dCMP and Me-dCMP was similar in different rat tissues (Table I); both activities were destroyed at the same rate during preincubation of a liver preparation at high temperatures (Fig. 1A) or at extreme pH values (Fig. 1B). Consequently it would appear that only one deaminase is involved in the conversion of dCMP

to thymidylate. In all subsequent experiments, dCMP was used as substrate for the deaminase assay.

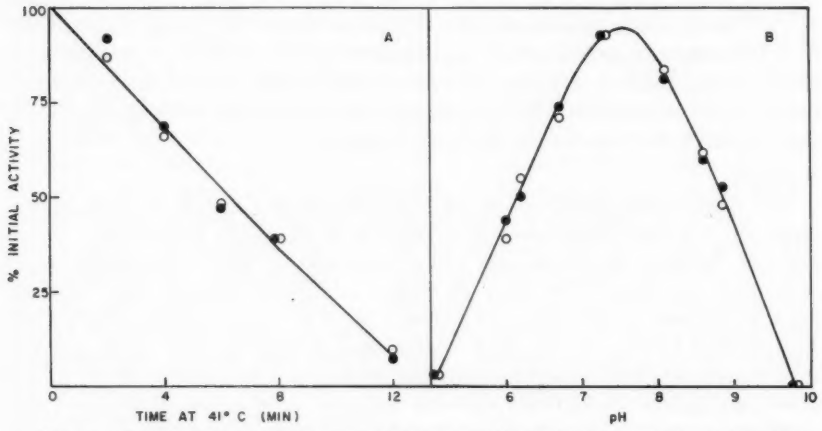

FIG. 1. Inactivation of deaminase activities towards dCMP (○) and Me-dCMP (●) following incubation of supernatants from regenerating liver homogenates: (A) at 41° C and pH 7.3 for varying periods of time; (B) at 20° C and varying pH values for 60 minutes.

Deaminase Levels in Normal and Regenerating Rat Liver

Deaminase activity was high in the liver of female rats shortly after birth but fell steadily as the animal matured (Table II). This decrease in activity parallels the established decrease in rate of cell proliferation in the liver of growing animals. Despite the decrease in activity per gram of liver, the total activity per liver continued to increase slowly after birth until the animals reached 6 weeks of age (Table II).

TABLE II

Deoxycytidylate deaminase levels in normal liver from female rats of different ages

Age of rat (days)	Number of rats	Average body weight (g)	Average liver weight (g)	Deaminase activity	
				μmoles dCMP/ g/hour	μmoles dCMP/ liver/hour
2	8	7	0.29	3.8	1.1
9	6	15	0.45	3.1	1.5
20	3	35	1.37	2.1	2.8
34	4	100	4.55	1.9	8.8
42	5	129	5.73	1.7	10.2
57	3	167	7.20	1.0	7.4
70	8	179	7.76	0.8	6.1

Following partial hepatectomy of female rats, deaminase activity in the regenerating liver rose to a high level on the second and third day after the operation, followed by a sharp drop to a lower level on the fourth day (Table III).

TABLE III

Deoxycytidylate deaminase levels in regenerating rat liver

Time after operation (days)	Deaminase activity (μmoles dCMP/g/hour)				Total deaminase content (μmoles dCMP/liver/hour)			
	A	B	C	D	A	B	C	D
0	1.9±0.1(4)	0.8±0.1(8)	0.8±0.1(8)	0.8±0.1(8)	2.8	1.9	1.9	6.1
1	2.6±0.9(4)	1.6±0.3(6)	1.4±0.3(2)	1.2±0.1(4)	5.4	5.3	4.2	8.0
2	6.2±0.5(6)	5.6±0.2(5)	5.9±0.4(4)	1.5±0.2(4)	16.8	25.3	22.4	9.8
3	8.4±0.9(4)	6.4±0.5(10)	5.0±1.1(8)	1.0±0.2(4)	29.4	36.7	19.9	6.7
4	—	2.5±0.4(6)	1.2±0.2(3)	—	—	14.8	4.6	—

NOTE: Average values for deaminase activity are given together with the standard error of the mean, followed by the number of animals in parenthesis. The four groups of animals are designated as follows: (A) 5-week-old female rats, partial hepatectomy, fed; (B) 10-week-old female rats, partial hepatectomy, fed; (C) 10-week-old female rats, starved after partial hepatectomy; (D) 10-week-old female rats, laparotomy.

Although the initial activity in normal liver was 2.4 times higher in 5-week-old than in 10-week-old animals, the maximum activity in regenerating liver was approximately the same for animals of both ages (Table III). Thus the relative increase in enzyme activity during liver regeneration was dependent on the age of the animal.

A sham operation was sufficient to produce a moderate increase in liver deaminase activity (Table III). However, part of this increase could probably be ascribed to loss of liver weight during recovery from the operation. The total amount of deaminase in the liver increased only 60% following a sham operation, but increased 1830% following partial hepatectomy of 10-week-old rats.

Figure 2 illustrates a comparison between the present data on deaminase levels and previous data (8, 9) on net DNA synthesis during liver regeneration. Deaminase activity and DNA synthesis in the liver appeared to increase in a similar manner up to the second day after partial hepatectomy of control animals. However, DNA synthesis decreased sharply between the second and

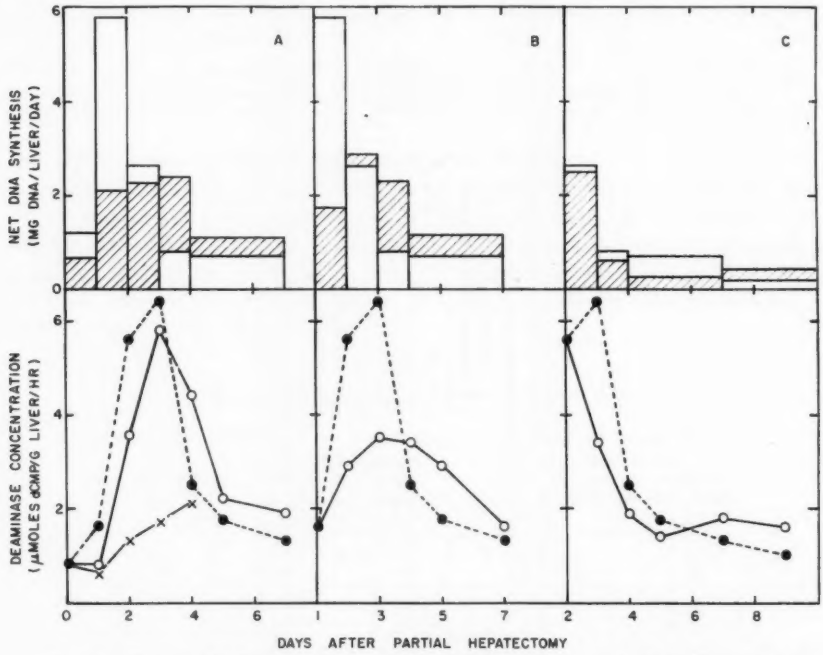

FIG. 2. Deoxycytidylate deaminase activity and net DNA synthesis in regenerating livers from control rats and from rats irradiated (A) just before partial hepatectomy, (B) 1 day after partial hepatectomy, and (C) 2 days after partial hepatectomy.

In the bar diagram section of the figure the open portion represents DNA synthesis in control animals and the cross-hatched portion represents DNA synthesis following 600 r X irradiation; ● deaminase concentration in control animals; ○ deaminase concentration following 600 r X irradiation; × deaminase concentration following 1500 r X irradiation.

third day after the operation, while the deaminase activity continued to increase slightly during the same period. Analogous results were obtained by Bollum and Potter (2) with the multienzyme system involved in the in vitro incorporation of thymidine into DNA.

Effect of X Irradiation on Deaminase Levels in Regenerating Liver

Exposure of the liver region to 600 r did not have any immediate effect on the deaminase activity of the liver of non-hepatectomized rats; 20 hours after irradiation, deaminase activity was 0.7 ± 0.1 μmole/g/hour as compared with 0.8 ± 0.1 μmole/g/hour in control animals. However, the usual increments in deaminase activity and in DNA synthesis during liver regeneration were delayed when the liver region was irradiated just before partial hepatectomy (Fig. 2A). In both cases, 1500 r produced more drastic effects than 600 r. The effects of irradiation cannot be ascribed simply to inanition, since starvation of animals following partial hepatectomy did not prevent the usual increase in deaminase activity of the liver (Table III). For comparison, the changes in average liver weight are shown in Fig. 3. As in previous experiments (8, 9), exposure of the

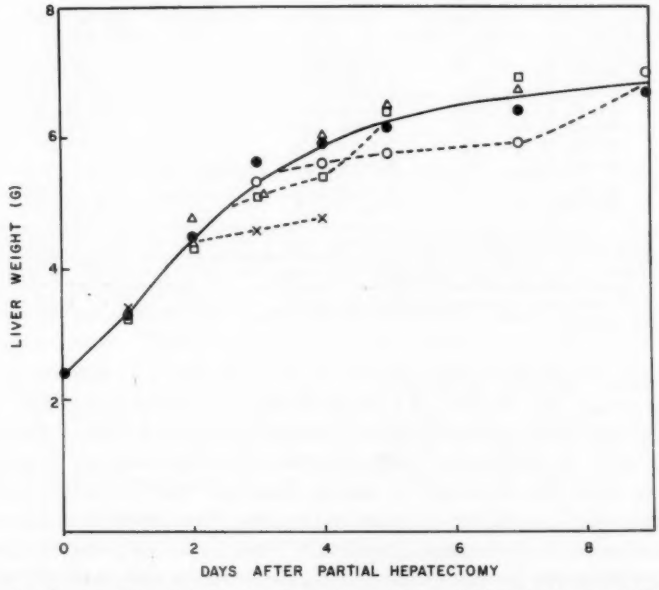

FIG. 3. Average liver weights at various times after partial hepatectomy and X irradiation. ● Controls; × 1500 r on day 0; □ 600 r on day 0; △ 600 r on day 1; ○ 600 r on day 2.

liver region to either 600 r or 1500 r had no immediate effect on the increase in total weight during liver regeneration, although a lag did develop after the second day, particularly following 1500 r.

The effect of 600 r X irradiation 1 day after partial hepatectomy was generally similar to the effects described above; again the increase in deaminase activity was retarded but not prevented (Fig. 2B). Further experiments at shorter intervals of time after irradiation confirmed this conclusion and showed that the deaminase levels in regenerating liver did not decrease significantly at any time within the first 25 hours following irradiation (Table IV). The last two experiments shown in Table IV were of particular interest; these results indicated that 600 r X irradiation did not prevent the usual increment in deaminase levels within the first 5–11 hours after irradiation. A significant effect on deaminase levels only became apparent later than 11 hours after exposure to 600 r.

TABLE IV

Deoxycytidylate deaminase activity of regenerating rat liver following 600 r X irradiation on the first day after hepatectomy

Time of irradiation	Time and day killed	Hours after irradiation	Deaminase activity (μmoles dCMP/g/hour)			P_1	P_2
			Control	Sham irradiation	Irradiated		
—	09.30, I	—	1.6±0.3(6)	—	—	—	—
08.00	13.00, I	5	2.2±0.6(3)	—	2.1±0.2(3)	—	—
07.30	18.30, I	11	3.1±0.4(3)	—	2.3±0.6(3)	—	—
07.30	23.30, I	16	4.5±0.4(5)	4.1±0.5(3)	2.8±0.4(4)	*	*
08.00	06.00, II	22	5.1±0.3(4)	5.4±0.4(3)	3.1±0.5(3)	*	*
07.30	09.00, II	25.5	5.6±0.2(5)	—	2.9±0.2(5)	**	**
07.30	09.30, III	50	6.4±0.5(10)	6.2±0.2(3)	3.5±0.3(6)	**	**
18.30	23.30, I	5	4.5±0.4(5)	4.2±0.4(3)	4.7±0.5(3)	*	—
13.30	23.30, I	11	4.5±0.4(5)	—	4.0±0.5(3)	*	—

NOTE: Average values for deaminase activity are given together with the standard error of the mean, followed by the number of animals in parenthesis. The day is indicated in Roman numerals, where I represent the first day after partial hepatectomy. P_1 indicates the statistical significance of the increase in deaminase activity of irradiated livers as compared with the deaminase activity in control livers at the time of irradiation; P_2 indicates the statistical significance of the decrease in deaminase activity of irradiated livers as compared with the deaminase activity in control livers at the time of death. In both cases, * represents a probability of 0.05 or less, while ** indicates a probability less than 0.01.

A different result was observed following irradiation with 600 r 2 days after partial hepatectomy; deaminase activity in the regenerating liver fell abruptly within the next 24 hours and failed to increase again until 5 days after irradiation (Fig. 2C). A subsequent series of experiments was designed to investigate this fall in deaminase activity at shorter intervals than 24 hours (Table V). During this study, a diurnal variation in the deaminase activity of regenerating liver of control animals became apparent.* This diurnal pattern, which seemed to be superimposed on the general changes in deaminase activity described previously, was evident between the second and fourth days after partial hepatectomy. Deaminase activity in control regenerating livers tended to remain fairly constant during the late morning and afternoon, to rise during the night, and to fall abruptly in the early morning (Table V). Despite this

*Recent data reported by Maley and Maley (18) show a similar diurnal variation in deaminase activity but not in thymidylate synthetase activity.

TABLE V

Deoxycytidylate deaminase activity of regenerating rat liver following 600 r X irradiation on the second day after partial hepatectomy

Time of irradiation	Time and day killed	Hours after irradiation	Deaminase activity (μmoles dCMP/g/hour)			P_2
			Control	Sham irradiation	Irradiated	
08.00	09.00, II	1	5.6±0.2(5)	5.8±0.5(2)	6.0±0.4(4)	—
08.00	13.00, II	5	6.0±0.8(3)	5.9±0.2(2)	5.3±0.7(5)	—
07.30	18.30, II	11	5.7±0.4(4)	5.2±0.6(3)	5.1±0.3(6)	—
07.30	23.30, II	16	6.6±0.9(5)	6.1±0.4(3)	4.0±0.2(6)	**
07.30	03.30, III	20	6.9±0.8(3)	7.3±1.0(3)	3.7±0.5(4)	**
07.30	09.30, III	26	6.4±0.5(10)	6.0±0.5(3)	3.4±0.6(6)	**
07.30	18.30, III	35	6.4±0.6(3)	—	3.3±0.5(3)	**
07.30	23.30, III	40	6.3±1.4(3)	—	3.3±0.2(3)	*
07.30	06.30, IV	47	5.5±0.9(4)	—	2.5±0.3(3)	*
07.30	09.30, IV	50	2.5±0.4(6)	—	1.9±0.6(3)	—
18.30	23.30, II	5	6.6±0.9(5)	6.4±0.7(4)	6.6±0.5(7)	—
15.30	23.30, II	8	6.6±0.9(5)	—	6.3±0.8(4)	—
12.30	23.30, II	11	6.6±0.9(5)	6.9±0.8(3)	4.6±0.2(4)	*
15.30	09.30, III	18	6.4±0.5(10)	6.8±0.8(4)	3.9±0.3(10)	**
23.00	07.00, III	8	7.4±0.4(6)	—	6.9±0.3(3)	—
23.00	10.00, III	11	—	6.9±0.9(3)	6.0±0.5(3)	—
23.00	15.00, III	16	5.9±0.5(4)	6.1±0.5(3)	3.4±0.4(4)	**
23.00	18.30, III	19.5	6.4±0.6(3)	—	3.2±0.3(3)	**

NOTE: All symbols are the same as given in Table IV.

complication, the results showed that a significant decrease in deaminase activity appeared consistently 11–16 hours after 600 r X irradiation. Once this decrease had occurred, deaminase activity in the irradiated livers was found to remain unchanged for some time (Table V). These effects were still evident when total deaminase activity per liver was used as the basis of comparison in order to compensate for any changes in liver weight (Fig. 4).

Irradiation with 170 r on the second day after partial hepatectomy was apparently sufficient to produce a maximal decrease in deaminase activity; the results obtained 18 hours after exposure to 170, 600, or 1500 r did not differ significantly (Table VI). Exposure to 50 r, on the other hand, was much less effective, while sham irradiation had no significant effect on the deaminase activity of regenerating liver at any time (Tables IV, V, and VI).

Effect of Other Antimitotic Agents on Deaminase Levels

It seemed probable that the diurnal variations in deaminase activity of regenerating liver (Fig. 4) were related to the diurnal variation in rates of DNA synthesis and mitosis which have been described previously (10–12). In an effort to determine whether cell division necessarily caused a change in the deaminase activity of dividing cells, we treated the experimental animals with two mitotic inhibitors: colchicine (13, 14) and normal rat serum (15). However, deaminase levels in regenerating liver were not significantly altered at any time by intraperitoneal injection of serum from adult rats and were only slightly

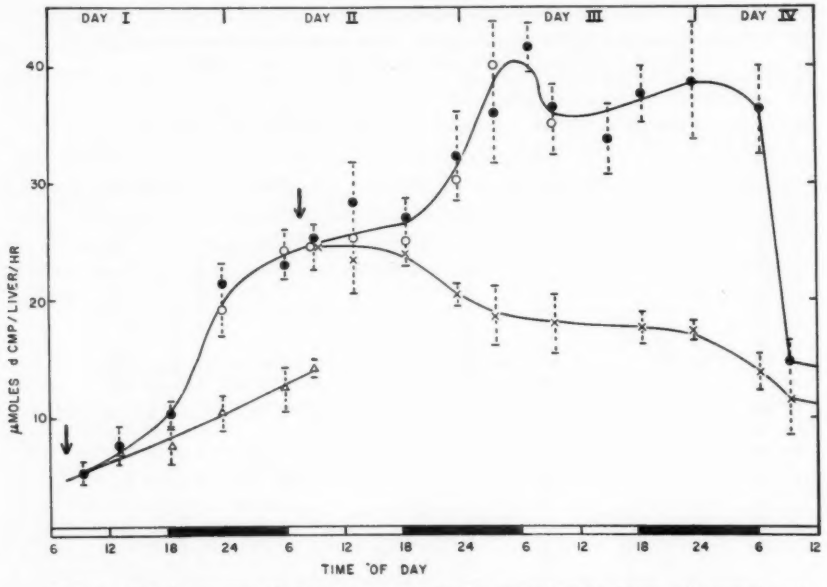

FIG. 4. Total amount of deaminase activity in regenerating livers of control and X-irradiated rats. ● Control animals; ○ sham-irradiated animals; △ animals exposed to 600 r at the time indicated by the first arrow on day I; × animals exposed to 600 r at the time indicated by the second arrow on day II. Standard errors of the mean values are represented by vertical interrupted lines. Periods of light (▭) and darkness (▬) are indicated at the bottom of the graph.

TABLE VI

Decrease in deoxycytidylate deaminase activity of regenerating rat liver following different doses of X radiation

Dose of X radiation	Deaminase activity (μmoles dCMP/g/hour)	Total deaminase content (μmoles dCMP/liver/hour)
None	6.4±0.5(10)	36.7±2.8(10)
Sham irradiation	6.8±0.8(4)	38.0±3.1(4)
50 r	5.6±0.5(3)	30.8±3.7(3)
170 r	4.0±0.9(5)*	21.3±2.9(5)**
600 r	3.9±0.3(10)**	20.8±1.8(10)**
1500 r	4.4±0.4(5)*	23.7±3.1(5)**

NOTE: The animals were irradiated at 14.30–15.30 on day II and killed for deaminase assay at approximately 09.30 on day III, i.e., 18–19 hours later. The asterisks indicate the decrease in deaminase levels following irradiation is statistically significant, as indicated in Table IV.

altered by injection of 0.3 mg of colchicine/kg 18–19 hours before the animals were killed for deaminase assays (Table VII). Both of these treatments should be sufficient to block normal cell division in the regenerating livers during this period of time (13–15). A higher dose of colchicine did delay the usual increase in deaminase activity during the first 2 days after partial hepatectomy (Table VII). However, even the higher dose of colchicine failed to produce a decrease

in deaminase activity between the second and third day after partial hepatectomy (Table VII), in contrast to the effects of X irradiation (Table VI). Furthermore, the same dose of colchicine failed to prevent the usual fall in deaminase activity between the third and fourth day after partial hepatectomy.

TABLE VII

Deoxycytidylate deaminase activity of regenerating rat liver following injections of adult rat serum or colchicine

Days after partial hepatectomy	Deaminase activity (μmoles dCMP/g/hour)			
	Control	15 ml serum/kg	0.3 mg colchicine/kg	1.0 mg colchicine/kg
0	0.8±0.1(8)	—	—	—
1	1.6±0.3(6)	2.1±0.2(3)	1.2±0.1(2)	0.9±0.3(2)
2	5.6±0.2(5)	5.6±0.9(5)	4.3±0.6(5)*	2.3±0.4(4)**
3	6.4±0.5(10)	6.6±0.7(6)	6.7±0.5(8)	5.8±0.7(6)
4	2.5±0.4(6)	3.0±0.5(3)	2.6±0.2(3)	2.0±0.3(3)

NOTE: All animals were injected at 14.30–15.30 and killed for deaminase assay at approximately 09.30 on the following day, i.e., 18–19 hours later. The asterisks indicate that the deaminase level of the treated rats is significantly lower than that of the control animals, as indicated in Table IV.

The failure of colchicine to affect deaminase levels in short-term experiments was confirmed by another series of experiments with 6-week-old, non-hepatectomized rats. Deaminase activities of liver and thymus were not significantly altered at 3, 6, 12, 18, and 26 hours after subcutaneous injection of 0.3 mg colchicine/kg.

Discussion

It was suggested previously that X irradiation of regenerating liver would interfere with the formation of one or more of the enzymes required for DNA synthesis, without directly affecting the enzyme activity already present in the liver at the time of irradiation (8, 16). This suggestion was confirmed by the present data on deoxycytidylate deaminase and those reported recently for the multienzyme system involved in the incorporation of thymidine into DNA (5). However, two unexpected effects of irradiation were observed. First, the deaminase activity continued to increase in a normal manner for 5–11 hours after exposure to 600 r during the early stages of liver regeneration; subsequently, the formation of new enzyme was retarded in the irradiated livers (Table IV and Fig. 4). Second, irradiation on the second day after partial hepatectomy, when deaminase levels in control regenerating livers were relatively constant, resulted in a decrease in deaminase activity to a new level 11–16 hours later (Table V and Fig. 4). Presumably these two phenomena are related, but the explanation of these results is still uncertain. One possible mechanism might be a selective effect of the radiation on some metabolic process which precedes the appearance of active enzyme in the liver by approximately 11 hours, or possibly irradiation results in a progressive damage which accumulates and reaches an effective level several hours later.

The observed decrease in deaminase activity 11–16 hours after exposure to 600 r on the second day after hepatectomy (Table V) is not necessarily inconsistent with the fact that net DNA synthesis during a 24-hour period was scarcely affected by irradiation under the same conditions (9). A comparison of deaminase levels and DNA synthesis on the second and third day after partial hepatectomy (Fig. 2) indicated that deaminase levels in control regenerating liver on the third day must have been considerably in excess of the minimum which might be required for the synthesis of DNA in vivo. Consequently a 40–50% decrease in deaminase levels following irradiation on the second day would still leave ample enzyme to provide for the relatively slow synthesis of DNA which did occur in irradiated, as well as in control, regenerating livers during this period of time. Moreover, deoxycytidylate deaminase may not be essential for DNA synthesis in parenchymal liver cells (17); other metabolic pathways for biosynthesis of thymidylate are known to exist.

The effects of different doses of radiation on deaminase activity in regenerating liver (Table VI) might be compared with their effects on cell division under the same conditions (9). A dose of 170 r, which was sufficient to produce a maximal decrease of approximately 40% in deaminase activity between the second and third day after partial hepatectomy, was also sufficient to produce a maximal effect on cell division, i.e., to prevent any increase in the number of cell nuclei during a 24-hour period. In both cases, 50 r was much less effective. These two effects may be due, therefore, to the same defect in the irradiated cells.

References

1. F. J. Bollum. J. Am. Chem. Soc. **80**, 1766 (1958).
2. F. J. Bollum and V. R. Potter. Cancer Research, **19**, 561 (1959).
3. R. Mantsavinos and E. S. Canellakis. J. Biol. Chem. **234**, 628 (1959).
4. G. F. Maley and F. Maley. J. Biol. Chem. **234**, 2975 (1959).
5. F. J. Bollum, J. W. Anderegg, A. B. McElya, and V. R. Potter. Cancer Research, **20**, 138 (1960).
6. S. Okada and L. H. Hempelmann. Intern. J. Radiation Biol. **1**, 305 (1959).
7. E. Scarano. J. Biol. Chem. **235**, 706 (1960).
8. D. K. Myers. Can. J. Biochem. and Physiol. **38**, 1059 (1960).
9. D. K. Myers. Can. J. Biochem. and Physiol. **38**, 1489 (1960).
10. J. J. Jaffe. Anat. Record, **120**, 935 (1954).
11. L. D. Liozner, Z. A. Ryabinina, and V. F. Sidorova. Byull. Eksptl. Biol. Med. **47**, 623 (1959).
12. C. P. Barnum, C. D. Jardetzky, and F. Halberg. Am. J. Physiol. **195**, 301 (1958).
13. A. Brues and E. Jackson. Am. J. Cancer, **30**, 504 (1957).
14. O. J. Eigsti and P. Dustin, Jr. Colchicine in agriculture, medicine, biology and chemistry Iowa State College Press, Ames, Iowa, U.S.A. 1955.
15. H. F. Stitch and M. L. Florian. Can. J. Biochem. and Physiol. **36**, 855 (1958).
16. R. E. Beltz and R. L. Applegate. Biochem. Biophys. Research Comm. **1**, 298 (1959).
17. H. C. Pitot and V. R. Potter. Biochim et Biophys. Acta, **40**, 537 (1960).
18. F. Maley and G. F. Maley. J. Biol. Chem. **235**, 2968 (1960).

RESOLUTION OF THE APOENZYME AND COENZYME OF L-GLUTAMIC ACID DECARBOXYLASE FROM SUNFLOWER COTYLEDONS[1]

J. E. SMITH[2] AND E. R. WAYGOOD

Abstract

Methods for the resolution of glutamic acid decarboxylase of sunflower cotyledons into the apo- and co-enzyme moieties are described. The apoenzyme can be separated from codecarboxylase by repeated acid – ammonium sulphate treatment with about 90% resolution and 40–50% inactivation. A codecarboxylase factor can be isolated as the barium salt following denaturation and precipitation of the protein by trichloracetic acid.

Introduction

During studies (1) on the activity of glutamic acid decarboxylase in healthy and rust-infected sunflower cotyledons it was found necessary to separate the apo- and co-enzyme moieties of this enzyme. The coenzyme is firmly bound to the protein of glutamic decarboxylase and rigorous procedures are required to separate the two components. The apoenzyme can be reactivated by the addition of pyridoxal phosphate.

This paper reports a procedure used for the resolution of the apoenzyme and also a procedure for the extraction and partial purification of a codecarboxylase from sunflower cotyledons which is probably identical with pyridoxal phosphate.

Materials and Methods

The seeds of the rust-susceptible inbred line of Sunflower (*Helianthus annuus* L. S.37-388 var. Foundation) were surface sterilized by shaking in a solution of 5% Javex for 15 minutes, followed by several washings in tap water. The seeds were sown in a mixture of loam and sand, 3:1 (v/v), and the cotyledons were harvested, after 6–7 days, from the greenhouse.

Glutamic acid decarboxylase was assayed by measuring CO_2 evolution at 30° C in a conventional Warburg apparatus. The main compartment of the flasks contained 1.0 ml of enzyme preparation with 150 μmoles phosphate buffer, pH 5.7, and 2 μmoles of pyridoxal phosphate except where otherwise stated. The reaction was started by tipping in 50 μmoles of sodium glutamate from the side arm. The final volume was 3.2 ml. The gaseous phase was air, since oxygen uptake of crude extracts was negligible. No correction was made

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for CO_2 retention at this pH. Specific activity is defined as $Q_{CO_2}^{(N)}$ = μl CO_2/mg protein/hr.

Protein determinations were made by a modification of the biuret method (2).

Results

Kinetic studies (1) indicated that the enzyme was similar to that demonstrated in several higher plant tissues (3, 4, 5, 6). The optimum pH was 5.7–5.8. The K_m value at 30° C was 9.1×10^{-3} *M* in comparison with 9.6×10^{-3} *M* for the enzyme from barley roots (3). The enzyme when resolved was reactivated by pyridoxal phosphate and the end product of the reaction, identified by paper chromatography, was γ-aminobutyric acid.

Resolution of the Apoenzyme

Crude undialyzed phosphate extracts from healthy cotyledons rapidly decarboxylate glutamic acid and respond slightly to additions of pyridoxal phosphate. Dialysis of the preparation against distilled water for 24 hours at 2° C had little effect on the activity indicating that the coenzyme is firmly bound to the protein. The enzyme precipitates in the fraction between 30 and 60% $(NH_4)_2SO_4$ saturation. By this step the specific activity is trebled. A second fractionation between the same $(NH_4)_2SO_4$ limits increases the specific activity sixfold with little or no change in the response of the enzyme to additions of pyridoxal phosphate. Table I shows the activity of the enzyme from

TABLE I

Purification of glutamic decarboxylase

	$Q_{CO_2}^{(N)}$			
	Healthy		Rust-infected	
Fraction	−PP	+PP	−PP	+PP
(1) Crude brei	510	546	276	414
(2) 1st 30–60% $(NH_4)_2SO_4$	1536	1638	900	1278
(3) 2nd 30–60% $(NH_4)_2SO_4$	3084	3276	1800	2760

NOTE: PP = Pyridoxal phosphate.

healthy tissue after two stages of purification compared with that of the enzyme from rust-infected tissue. The difference between the enzyme from healthy and rust-infected tissue is noticeable, but these preliminary purification steps remove little or none of the bound coenzyme in either case.

The enzyme was subsequently resolved into its specific apoenzyme moiety by a modification of the procedure used by Cori and Illingworth (7) for the dissociation of the pyridoxal phosphate of muscle phosphorylase.

Approximately 10 g of 7-day-old chilled cotyledons were ground in 10 ml of 0.2 *M* phosphate buffer, pH 5.7. After being pressed through cheesecloth, the filtrate was centrifuged at $20{,}000 \times g$ for 15 minutes and the supernatant was made to 20 ml with buffer. Three volumes of 60% saturated ammonium sulphate solution, containing 10% (v/v) ammonia (specific gravity = 0.88), were

added with constant stirring to give a final pH of 10.0. The solution was allowed to stand for 20 minutes in the cold and then it was adjusted cautiously with *N* HCl to pH 3.0 with rapid stirring. Pronounced precipitation of the enzyme occurred at pH 4.0 and the protein was collected by centrifugation. Assays indicated that little or no coenzyme had been removed by this preliminary treatment. The precipitate was then dispersed in a small quantity of 50% saturated ammonium sulphate solution, pH 8.5, and made up to 10 ml with this solution. It was stirred thoroughly and centrifuged at 15,000×*g* for 2 minutes. The precipitate was washed 3 times with 50% ammonium sulphate solution, pH 8.5, and finally dissolved in 20 ml 0.2 *M* phosphate buffer, pH 5.7. Approximately 35–40% of the enzyme had been dissociated from its coenzyme at this stage with about 10% inactivation of the holoenzyme (Table II, 1st acid treatment).

To obtain almost complete resolution it was essential to carry out the entire procedure 3 times, which resulted in 85–90% dissociation of the enzyme from the coenzyme with approximately 45% inactivation (Table II). The degree

TABLE II

Resolution of glutamic decarboxylase

Stage	$Q_{CO_2}^{(N)}$ −PP	$Q_{CO_2}^{(N)}$ +PP	Resolution (%)	Inactivation (%)
Initial extract	630	672	—	—
1st acid treatment	390	600	35	10
2nd acid treatment	204	510	60	24
3rd acid treatment	36	360	90	46

of resolution increases with acidity, but care must be taken at the acid precipitation stage that the precipitation, centrifugation, and dispersal in ammonium sulphate solution should be carried out in a period not in excess of 5 minutes. In these assays, the apoenzyme was reactivated with commercial pyridoxal phosphate.

Extraction of Codecarboxylase

Gale and Epps (8) extracted and partially purified a factor from yeast which was highly active as a coenzyme lysine and tyrosine apodecarboxylases. The active principle in this preparation, which was only 10% pure, is widely accepted as pyridoxal phosphate. No compound, apart from pyridoxal phosphate or its precursors, has been found to possess the property of being able to reactivate the amino acid apodecarboxylases. Although Gale and Epps (8) found a trace of coenzyme activity in pea leaves, their method was unsuccessful with sunflower cotyledons. Recently, Baranowski *et al.* (9) have isolated pyridoxal phosphate from muscle phosphorylase by precipitating the enzyme with trichloracetic acid and isolating the acid-soluble compound as the barium salt. The compound was identified by its spectral characteristics, paper electro-

phoresis, and specific enzymatic assays. By modifying their procedure we have been able to prepare an extract from sunflower cotyledons that reactivates the apoenzyme of squash and sunflower glutamic decarboxylase and bacterial tyrosine decarboxylase. Only a partial purification was attained, as follows.

Cotyledons were washed and ground with an equal volume of 0.2 *M* phosphate, pH 5.7. The brei was pressed through cheesecloth and the filtrate brought to room temperature on standing. Trichloracetic acid (50%) was added dropwise with rapid stirring to give a final concentration of 10%. The mixture was allowed to stand for 30 minutes and then centrifuged at 20,000×*g* for 10 minutes. The apoenzyme was inactivated by this treatment. The orange-yellow supernatant was decanted, stored at 2° C, and the precipitate was taken up in 0.2 *M* phosphate, pH 5.7. The trichloracetic acid extraction was repeated twice with extraction times of 30 minutes and 1 hour.

The three supernatant solutions were combined and washed with several portions of anhydrous ether until the reaction was faintly red to litmus. The aqueous solution was adjusted to pH 8.5 with freshly prepared 10% sodium hydroxide followed by the addition of an equal volume of 95% ethanol. The precipitate that formed was discarded since it was inhibitory to glutamic decarboxylase. An excess of barium acetate was added to the alcoholic supernatant fluid followed by the addition of another equal volume of 95% ethanol, and the resulting precipitate was allowed to settle overnight in the cold. The yellow supernatant fluid was discarded and the sludge separated by centrifugation at 10,000×*g* for 15 minutes. The codecarboxylase, which is in the form of an insoluble barium salt, was dissolved in 0.5 *N* H_2SO_4. After low-speed centrifugation the precipitate was re-extracted with 0.5 *N* H_2SO_4 and the two supernatants were combined and neutralized. This light brownish liquid was capable of reactivating the apodecarboxylases shown in Table III.

TABLE III

Codecarboxylase activity of extract from sunflower cotyledons

Apodecarboxylase	$Q_{CO_2}^{(N)}$		
	−PP	+PP	+extract
Glutamic apodecarboxylase			
Squash	144	540	360
Sunflower	60	450	300
Bacterial tyrosine apodecarboxylase	180	540	324

Attempts to identify the characteristic absorption bands of pyridoxal phosphate (295 mμ in acid and 405 mμ in alkaline solution) were unsuccessful owing to the masking effect of impurities. Possibly the concentration was too low since commercial pyridoxal phosphate (2 μmoles/ml) failed to reveal absorption bands when added to the extract. Some further purification was obtained when the extract was eluted from a Dowex 1-X-8 formate column by 0.5 *M* formic acid. Slight absorption was revealed at 295 mμ in acid, but the bulk of the

contaminating compounds were still present. Further purification steps starting with larger quantities of material are required for an unequivocal identification of the codecarboxylase as pyridoxal phosphate.

The procedure used by Holzer *et al.* (10) for the resolution of the apo- and co-enzymes of brewers yeast transaminase was not available to the authors at the time these studies were undertaken. However, they were able to dialyze a large portion of the bound coenzyme, a method which was always unsuccessful with the decarboxylase from our tissue. Their procedure for the extraction of pyridoxal phosphate from baker's yeast, which involves boiling neutralized trichloracetic acid precipitate, may provide a useful method for plant tissue.

References

1. J. A. SMITH. Alterations in the activity of certain enzymes involved in the nitrogen metabolism of resistant and susceptible sunflower varieties associated with infection by Puccinia helianthi Schw. Ph. D. thesis, University of Manitoba, Winnipeg, Manitoba. 1959.
2. A. G. GORNALL, C. J. BARDAUILT, and M. M. DAVID. J. Biol. Chem. **177**, 751 (1949).
3. H. BEEVERS. Biochem. J. **48**, 132 (1951).
4. S. L. HOOD. Botan. Gaz. **116**, 86 (1954).
5. O. SCHALES and S. SCHALES. Arch. Biochem. **11**, 155 (1946).
6. P. WEINBERGER and K. A. CLENDENNING. Can. J. Botany, **30**, 755 (1952).
7. C. F. CORI and B. ILLINGWORTH. Proc. Natl. Acad. Sci. **43**, 547 (1957).
8. E. F. GALE and E. EPPS. Biochem. J. **38**, 320 (1944).
9. T. BARANOWSKI, B. ILLINGWORTH, D. H. BROWN, and C. F. CORI. Biochim. Biophys. Acta, **25**, 16 (1957).
10. H. HOLZER, U. GERLACH, G. JACOBI, and M. GOTH. Biochem. Z. **329**, 529 (1958).

THE IN VITRO UTILIZATION AND PRODUCTION OF NON-ESTERIFIED FATTY ACIDS BY ADRENALECTOMIZED RATS[1]

W. F. Perry and Helen F. Bowen

Abstract

The in vitro utilization of non-esterified fatty acids by various tissues and the in vitro production of non-esterified fatty acids by adipose tissue have been compared in normal and adrenalectomized rats. It was found that the production of NEFA by adipose tissue was similar in both groups of animals but that the in vitro utilization of NEFA and production of carbon dioxide by heart, diaphragm, kidney, and liver tissue was greater in the adrenalectomized animal. These findings together with the depletion of fat content of the depots are interpreted as indicating that in the adrenalectomized state there is increased peripheral utilization of fatty acids.

There is a certain amount of evidence indicating that in the adrenalectomized animal there is an increased catabolism of fats. For example, both Engel and Engel (1) and ourselves (2) have shown that ketogenesis is increased in the adrenalectomized rat; accompanying this is a marked decrease in depot fat. In addition, Levy and Ramey (3) have recently shown that the fasted adrenalectomized rat can move more fat out of the depot than the fasted normal rat. We have also previously established that adipose tissue from adrenalectomized animals, although low in fat, has a much increased ability to synthesize fatty acids from acetate. This phenomenon is not present in animals 1 week after adrenalectomy but is very marked 2 or more weeks following removal of the adrenals. This, together with the apparent ability of the liver from the adrenalectomized rats to metabolize fatty acids at a greater than normal rate, led us to suggest (2) that in the absence of adrenal cortical hormones there was enhanced utilization of fatty acids by the liver and possibly by other tissues. It was in order to test this hypothesis that the following experiments were done.

Methods

Adult rats weighing 200–250 grams were used as experimental animals, either 1 week or 2 weeks following adrenalectomy. During the postoperative period they were given 1% NaCl as drinking water and allowed free access to food (Purine fox chow). Control non-operated rats were fed likewise and allowed water ad libitum. Twenty-four hours before killing, food was withdrawn from both control and operated animals. The rats were killed by decapitation, and the liver, heart, diaphragm, kidney, perirenal, and epididymal adipose tissues were quickly removed and placed in buffer. Slices, approximately 500 mg in weight, of heart, liver, and kidney were prepared and placed in 5-ml volumes

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Contribution from the Department of Physiology, University of Manitoba, and the Department of Biochemistry, Winnipeg General Hospital, Winnipeg, Manitoba.

of appropriate buffers for incubation. The diaphragm and epididymal fat pad were placed intact in 5 ml of incubation media. Perirenal tissue was macerated with scissors while in the buffer; pieces of the tissue were then removed, weighed, and placed in 5 ml of incubation medium. The various tissues were incubated at 37° C for 2½ hours in each of the following media: heart, kidney, diaphragm, and liver in phosphate buffer, at pH 7.4, containing potassium ion 70 meq/l., and sodium 76 meq/l.; the buffers also contained magnesium 6 meq/l., albumin 2.5%, and palmitic acid 0.60 meq/l. The added palmitic acid was labelled with C^{14} on the carboxyl carbon to give a concentration of 60 μc/l. The gas phase in the incubation flasks was 100% oxygen. An aliquot of the C^{14} palmitate was combusted; the CO_2 produced was counted as an infinitely thick plate of $BaCO_3$ (4, 5). The total counts of the palmitic acid present in each preparation was then calculated. It equalled 79,000 c.p.m. A similar buffer was used for adipose tissue except that albumin was added at a concentration of 12.5% and no palmitic acid was added. However, the albumin used had fatty acid combined with it giving a concentration of non-esterified fatty acid in the medium of approximately 1.4 meq/l.

Both before the addition of the tissues and at the end of the incubation period the media were analyzed for non-esterified fatty acids (NEFA) by Dole's (6) method and from the values obtained the amount of NEFA removed from (in the case of heart, kidney, diaphragm, and liver), or added to (adipose tissue), the incubation media was calculated. It has been alleged (11) that, with Dole's method for NEFA, lactic acid and phospholipid interfere significantly, giving factitiously high values. We have been unable to confirm this. The CO_2 produced during the incubation period was trapped in alkali and made up to volume; an aliquot was taken and the carbonate precipitated as barium carbonate, which was plated and counted as plates of infinite thickness (4, 5). The counts of the total sample was then calculated to give a measure of the oxidation of the fatty acids by the tissues.

The results were analyzed statistically using the "*t*" test for unpaired data.

Results

It will be seen from Table I that heart, diaphragm, kidney, and liver tissue from adrenalectomized animals removed more NEFA per gram of tissue from the incubation medium than did the same tissues from control animals. The results were similar whether the tissues were from animals 1 or 2 weeks after adrenalectomy. Since the absolute weights of the organs, when they could be accurately measured (heart and kidney), were little different in the control and experimental animals, it follows that the increased removal of palmitate from the medium reflects an increased utilization of fatty acids by these tissues. Further evidence of this increase is seen in the increased incorporation of the labelled carbon of the palmitic acid into CO_2 by the various tissues of the adrenalectomized animal (see Table II).

TABLE I

Utilization of palmitic acid in vitro by various tissues during 2½-hour incubation period
(Values expressed as μmoles palmitic acid/g tissue ±S.E.)

	No.	Heart	Wt. (mg)	Diaphragm	Kidney	Wt. (mg)	Liver
Controls	8	1.10 ±0.12	531	1.68 ±0.11	2.30 ±0.13	1,033	0.56 ±0.16
1 week adrenalectomized	8	1.73 ±0.24	532	2.72 ±0.16	2.70 ±0.15	1,020	1.80 ±0.25
P value		= .05		< .01	= .05		< .01
2 weeks adrenalectomized	8	1.91 ±0.20	526	2.60 ±0.19	2.85 ±0.17	1,032	1.52 ±0.13
P value		< .02		< .01	= .05		< .01

TABLE II

Production of $C^{14}O_2$ in vitro by various tissues incubated for 2½ hours in medium containing C^{14} palmitic acid (Values expressed as c.p.m./g tissue ± S.E.)

	No.	Heart	Diaphragm	Kidney
Controls	8	8,000 ± 1,200	6,500 ± 650	15,100 ± 850
1 week adrenalectomized	8	9,700 ± 1,390	8,200 ± 500	18,200 ± 970
P value		> .05	= .05	< .05
2 weeks adrenalectomized	8	11,700 ± 1,300	8,360 ± 580	19,250 ± 1,200
P value		= .05	= .05	< .05

From the data in Table III it will be noted that the total release of non-esterified fatty acids into the medium by adipose tissue was of similar magnitude in both control and experimental animals when referred to the wet weight of the tissue; but when expressed per milligram of adipose tissue nitrogen, the release by tissue from adrenalectomized animals was significantly less than by tissue from the controls.

TABLE III

Release of NEFA into medium by adipose tissue incubated 2½ hours in phosphate buffer

		Perirenal fat		Epididymal fat	
	No.	μmoles/g ± S.E.	μmoles/mg N ± S.E.	μmoles/g ± S.E.	μmoles/mg N ± S.E.
Controls	8	3.4 ± 0.32	2.00 ± 0.17	3.9 ± 0.42	2.37 ± 0.26
1 week adrenalectomized	8	3.0 ± 0.26	0.76 ± 0.12	3.2 ± 0.38	0.94 ± 0.16
P value		> .05	< .001	> .05	< .001
2 weeks adrenalectomized	7	3.0 ± 0.29	0.68 ± 0.08	3.8 ± 0.40	1.01 ± 0.13
P value		> .05	< .001	> .05	< .001

Discussion

In a previous report we demonstrated that liver tissue from adrenalectomized rats converted fatty acid to ketone bodies to a greater extent than liver tissue of normal animals. The depot fats of these adrenalectomized animals were markedly depleted of fat, but had a much increased ability to incorporate acetate into fatty acids. This led us to suggest that there was a more rapid turnover of fatty acid with an increased rate of mobilization of fat from the depots in such animals. In line with this concept is the finding of Levy and Ramey (3) that in the fasting adrenalectomized rat there is increased mobilization of total fat from the depots.

The present data have some bearing on the reason for the increased mobilization. They show that when animals are in the adrenalectomized state, various peripheral tissues in addition to liver have an enhanced rate of uptake and

oxidation of non-esterified fatty acids to CO_2, while at the same time fatty acid release in vitro by adipose tissue may be less than normal. It would thus appear that adrenalectomy depletes the fat depots due to the excessive utilization of fat in other tissues, and not to some abnormality in the depots themselves. We have previously shown (2) that in the adrenalectomized rat there is no increased oxidation of fatty acid by adipose tissue.

It is possible that these differences could be due to reduced food intake by the adrenalectomized animal. However, in a previous communication (7) we showed that fatty metabolism of the adrenalectomized rat differed from that of control animals regardless of whether the control animals were pair fed with the adrenalectomized animals or not.

Various workers have demonstrated that fatty livers in adrenalectomized animals, unlike those of normal animals, are not produced as a result of cold exposure, prolonged fasting (8), or treatment with epinephrine (9); nor is the epinephrine-induced rise in concentration of plasma NEFA found in adrenalectomized animals (10). These defects have been ascribed to defective mobilization of fatty acids from the depots. From the present work, these findings are also explicable on the basis of increased fatty acid utilization by various peripheral tissues.

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References

1. M. ENGEL and F. L. ENGEL. Endocrinology, **55**, 593 (1954).
2. W. F. PERRY and H. BOWEN. Am. J. Physiol. **189**, 433 (1957).
3. A. C. LEVY and E. R. RAMEY. Endocrinology, **64**, 586 (1959).
4. W. D. ARMSTRONG and J. SCHUBERT. Anal. Chem. **20**, 270 (1948).
5. C. ENTENMAN, S. R. LERNER, I. L. CHAIKOFF, and W. G. DAUBEN. Proc. Soc. Exptl. Biol. Med. **70**, 364 (1949).
6. V. DOLE. J. Clin. Invest. **35**, 150 (1956).
7. W. F. PERRY and H. BOWEN. Am. J. Physiol. **184**, 59 (1956).
8. L. LEVIN and R. K. FARBER. Recent Progr. in Hormone Research, **7**, 399 (1952).
9. E. M. MACKAY. Am. J. Physiol. **120**, 361 (1937).
10. E. SHAFRIR and D. STEINBERG. J. Clin. Invest. **39**, 310 (1960).
11. D. TROUT, E. H. ESTES, and S. FRIEDBERG. J. Lipid Research, **1**, 199 (1960).

PROBLEMS INVOLVED IN THE PURIFICATION OF ANTIHAPTEN ANTIBODIES BY THE USE OF STROMA–HAPTEN CONJUGATES[1]

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Abstract

Stroma-*p*-arsanilic acid conjugates were used for the isolation of antiarsanilic acid antibodies. The purity of antibody preparations obtained by displacement of antibodies with hapten was of the order of 60%. The recovery of antibodies was also about 60%. The reason for these values being lower than those theoretically possible was attributed to hapten inhibition.

Introduction

A large number of studies have been devoted to the development of general methods for the specific isolation of antibodies. For the isolation of antibodies to haptens, Landsteiner and van der Scheer (1) were the first to use specific adsorbents, which were prepared by coupling of the homologous haptens to red cell stroma and dissociation of the adsorbed antibodies with acid. This procedure led to antibody preparations which were only 50% pure, as determined by the precipitation of the recovered antibodies with appropriate protein–hapten conjugates. A similar method was employed by Eisen and Karush (2), who obtained preparations with a purity of 90%.

Campbell *et al.* (3) used a polyhaptenic azo dye, which was a trisubstituted derivative of resorcinol, for the precipitation of the homologous antibodies. The antibodies were then dissociated from the dye by addition of an excess of the free hapten. The dye was precipitated at pH 3.5 and any dissolved free hapten was removed by dialysis. By this procedure antibodies were obtained in a yield of 90% and a purity of 90%. However, Epstein *et al.* (4), using the same procedure, reported yields of only 45%. Karush and Marks (5) developed an alternate method by coupling haptens to fibrinogen, a protein which is less soluble than the antibody globulins. The antibodies to haptens were first precipitated by the addition of fibrinogen–hapten conjugates and subsequently dissociated off with a solution of free hapten. The fibrinogen–hapten conjugates were rendered insoluble in the presence of hapten and 0.05 *M* phosphate. The yield of antibody was 50% but the degree of purity of the antibody preparation was not indicated.

For the isolation of antibodies to protein antigens with specific immunosorbents, a number of insoluble substances such as cellulose (6, 7), ion exchange resins (8), and polystyrene (9, 10) were used as supporting media for the

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[2]Holder of N.R.C. Studentship.

appropriate antigens. In most of the procedures, the antibodies were eluted from the immunosorbents by dissociation under acid conditions, and according to Campbell *et al.* (6) this procedure led to the preparation of antibodies in a yield of 90% and a purity of 90%. However, the purity and yield in the other studies quoted were considerably lower.

In the present study, red cell stroma – hapten conjugates were used as the immunosorbent for the isolation of antiarsanilic rabbit antibodies. Since in an independent study in this laboratory (9) dissociation of antibodies from polystyrene–antigen conjugates at low pH resulted in an apparent modification of the antibodies, in the present investigation dissociation with an excess of sodium arsanilate was employed. An account of the problems encountered in the preparation of purified antihapten antibodies is given here.

Methods and Materials

Preparation of the Stroma–Hapten Conjugates

A procedure essentially similar to that of Landsteiner and van der Scheer (1) was used. Bovine erythrocytes were lysed by heating at 60° C for 1 hour, were then suspended in five volumes of water, and were shaken vigorously for 15 minutes. Enough sodium chloride was added to the suspension to make a 1% solution. The stroma was then separated by centrifugation. The stroma was repeatedly resuspended in saline solution and centrifuged until the supernatant obtained was colorless. For removal of lipids the stroma was resuspended in an equal volume of water to which half a volume of toluene was added. The mixture was shaken intermittently over a period of 48 hours and was then centrifuged. The toluene layer containing some of the lipids was discarded, and the stroma was freeze-dried. Subsequently it was extracted with ether at room temperature during 24 hours, and finally with ethanol in a Soxhlet extractor. The extracted stroma was dried by lyophilization.

For the coupling of arsanilic acid to the stroma, 0.5 millimole of diazotized *p*-arsanilic acid was used per gram of dried stroma. The latter was dispersed in 100 ml of water using a "Virtis" homogenizer and a volume of 18 ml of a 1 *M* sodium carbonate solution was added. The suspension was placed in an ice bath and the diazotized *p*-arsanilic acid was added dropwise, with vigorous stirring of the mixture for 1 hour. The suspension was then acidified with hydrochloric acid to pH 3.0 and centrifuged. The stroma–hapten conjugates were resuspended in borate buffer, centrifuged, and washed with the buffer (pH 8.0, $\Gamma/2 = 0.15$) at room temperature until the supernatant was colorless.

Preparation of Soluble Hapten–Protein Conjugates

On the average, about 40 *p*-arsanilic acid residues were coupled per molecule of bovine gamma globulin (BGG) or per molecule of human serum albumin (HSA), using standard diazotization and coupling procedures. The resulting protein–hapten conjugates (BGG-R and HSA-R) were dialyzed exhaustively against a solution of bicarbonate. Both conjugates were finally dialyzed against borate buffer.

Preparation and Isolation of Antibody

Rabbits were immunized intravenously 3 times a week for periods of 4–6 weeks with 1-ml portions of 2% solution of BGG-R until sufficiently high-titered antisera were produced. The rabbits were bled 5 days after the last injection. After a rest period of 3–4 weeks the immunization procedure was repeated. The antisera were Seitz-filtered into sterile vials and stored at 4° C.

For the isolation of antibodies, decomplemented sera were used. For removal of complement from anti-BGG-R sera an equal volume of BGG solution, containing enough BGG to precipitate all the anti-BGG antibodies, was added. Subsequently, approximately 0.1 g of packed, wet stroma–hapten conjugate was added to the antiserum, containing about 1 mg of antibody, and the suspension was stirred slowly at 4° C for 4 hours. The immunosorbent–antibody complexes were packed by centrifugation at 2000 r.p.m. for 10 minutes. They were washed with cold borate buffer. For the dissociation of anti-R antibodies, the immunosorbent–antibody complexes were suspended in a 5–10% solution of the sodium salt of *p*-arsanilic acid or the sodium salt of *p*-iodobenzene arsonic acid. The volume of dissociating hapten was equal to that of the antiserum used. The supernatants (hereafter referred to as eluates) were dialyzed against borate buffer (pH 8.0, $\Gamma/2 = 0.15$) in standard Visking tubing for 1 to 7 days. It was found necessary to boil the Visking tubing in distilled water for a few minutes to remove nitrogenous materials present in the interstices of the tubing. In an alternate method the antibodies were dissociated off the immunosorbents by adding hydrochloric acid to the suspension to a final pH of 3.2. The pH of the eluate was adjusted to 7.0 and the latter was dialyzed against borate buffer for 12–16 hours.

The antibody content of the whole sera and of the eluates was determined by the quantitative precipitin test, as recommended by Heidelberger and Kendall (11), and the nitrogen content of the precipitates was obtained by the micro-Kjeldahl procedure of McKenzie and Wallace (12).

Preparation of p-Iodobenzene Arsonic Acid

p-Iodobenzene arsonic acid was prepared according to the classical Sandmeyer reaction (13) and was recrystallized from 50% ethanol (14). For the preparation of I^{131}-labelled compound the recommendations of Sloviter (15) were used to destroy any free iodine produced in the reaction. The radioactivity of the samples was measured in a well type NaI (Tl) crystal scintillation counter and the specific activities ranged from 2.5×10^6 to 3.5×10^6 counts per minute per milligram.

Hapten-Inhibition Experiments

To two equal portions of decomplemented anti-R antiserum optimal concentrations of HSA-R were added and the mixtures were incubated at 4° C for 48 hours. The precipitates were washed with solutions of borate buffer to remove non-specific proteins. One of the precipitates was dissolved in an excess of sodium arsanilate. Both samples were transferred quantitatively into dialysis bags, and dialyzed for 72 to 96 hours against borate buffer. The amounts

of precipitate in both bags were measured. The same experiment was repeated using *p*-iodobenzene arsonic acid as an inhibitor.

Free Electrophoresis and Ultracentrifugation

Electrophoretic analyses of whole and absorbed sera were done in a Spinco–Tiselius apparatus. All experiments were performed in veronal buffer (pH 8.6, ionic strength 0.1) and the concentration of the various electrophoretic components was calculated from photographs of the Rayleigh interference fringe patterns of the ascending boundaries (16). An optical Spinco ultracentrifuge was used for the determination of the sedimentation properties of the various globulin preparations. For this purpose, samples of eluates were concentrated by dialysis against a 20% solution of polyvinyl pyrolidone (molecular weight 30,000) dissolved in normal saline. Before centrifugation the samples were dialyzed overnight against saline.

Results

The antibody concentration of the anti-R antisera used in this study ranged between 1 and 2 mg/ml. Comparison of the electrophoretic patterns obtained with the antisera prior to and after absorption with stroma–hapten conjugates showed that the bulk of the serum proteins removed were γ-globulins and that smaller amounts, corresponding to about 30% of the total materials removed, were α- and β-globulins. On the other hand, no albumin was removed on absorption. The results of a typical experiment are illustrated in Table I.

TABLE I

	Whole anti-R serum (g%)	Anti-R serum adsorbed with stroma-R (g%)
Albumin	3.45	3.45
α	0.94	0.91
β	0.78	0.75
γ	1.29	1.15

Ultracentrifugal analysis of the eluates revealed that most of the material (90%) had a sedimentation coefficient of 7 S, corresponding to that of normal γ-globulins, and that a small portion (10%) might have been some large molecular aggregate with a sedimentation coefficient of 20 S.

The results of absorption experiments demonstrated that complete removal of antibodies from homologous antisera was achieved and that about 0.1 g of wet, packed stroma–hapten conjugates was sufficient to combine with 1 mg of rabbit antibody. When antibodies were eluted with an excess of sodium arsanilate, the eluates were found to be colorless. This was taken as an indication that the hapten–stroma conjugates used in this study remained insoluble. The use of larger quantities of diazotized *p*-arsanilic acid in the coupling reaction, as originally employed by Landsteiner and van der Scheer (1), resulted in the production of large quantities of soluble stroma–hapten conjugates which

could not be completely removed from the insoluble conjugates on prolonged washing with buffer. Occasionally, however, small insoluble particles remained in suspension, and could not be removed on prolonged centrifugation at 2000 r.p.m., except after dialysis against borate buffer. This was probably caused by the high density of the solutions containing the large concentrations of sodium arsanilate used in these experiments.

Elution of anti-R-antibodies from the immunosorbent with sodium arsanilate, followed by dialysis of the eluates for removal of the hapten, demonstrated that the optimal yield of precipitable antibodies was obtained after dialysis for 72 hours. Thus, the recovery of precipitable antibodies after dialysis for 24 hours was about 30% and increased to 65% on increasing the period of dialysis to 72 hours. Dialysis for periods longer than 72 hours did not result in a further increase in the yeild or purity of the recovered antibodies (Table II).

TABLE II

Experiment No.	Time of dialysis (hours)	Total antibody in serum (mg/ml)	Total protein* eluted (mg/ml)	Precipitable antibody in eluate (mg/ml)	% yield	% purity
1	72	2.12	1.88	1.05	50	56
2	84	2.12	1.60	1.00	47	63
3	168	2.12	1.38	0.80	38	59
4	72	0.99	0.96	0.64	65	67
5	84	0.58	0.68	0.38	66	56
6	96	0.94	0.96	0.48	52	50

*These values were referred to the original volume of the serum used for absorption experiments.

These data were calculated on the basis of standard precipitin curves by subtracting the amount of HSA-R added from the total precipitate obtained at the optimal zone. The purity of the different antibody preparations, recovered after 72 hours of dialysis, which was computed in terms of precipitable antibodies and the total protein concentration of the eluates, ranged from 55 to 65%. Reabsorption of the eluate obtained in one experiment with fresh stroma-R conjugates resulted in the uptake of 92% of the total proteins. In contrast, in a control experiment treating normal rabbit γ-globulins[3] in an identical manner with an equal portion of the same immunosorbent preparation did not lead to any detectable removal of proteins. In another experiment the whole purification procedure was repeated with the eluates in an attempt to increase the purity of the final antibody preparation. This resulted in a further loss of antibodies without leading to a higher degree of purity.

The fact that the purity of the antibody preparations, as measured by the proteins precipitated with HSA-R, was lower than that calculated in terms of the material taken up by the immunosorbent on reabsorption (i.e. 65% as compared with 92%) was considered as an indication that complete precipita-

[3]Obtained by sodium sulphate fractionation according to the method of Marrack and Hoch (17).

tion of the eluted antibodies was inhibited by the eluting hapten which was strongly bound to some antibody molecules. To check this hypothesis, I^{131}-labelled *p*-iodobenzene arsonic acid[4] was used as the eluting hapten. The eluate was dialyzed for 72 hours. A solution containing similar amounts of normal rabbit γ-globulins and I^{131}-labelled *p*-iodobenzene arsonic acid served as control, and was dialyzed for the same length of time. From the difference in radioactivity of the two solutions it was calculated that 1 molecule of hapten was bound specifically per 2 molecules of γ-globulins in the eluate. Further confirmation that the low yields of precipitable antibody were attributable to inhibition by the hapten was obtained in "hapten inhibition" experiments (as described under Methods). The results of these experiments are given in Table III.

TABLE III

Hapten-inhibition experiments

	Hapten used	
	p-Arsanilic acid	*p*-Iodobenzene arsonic acid
	γ N in precipitate	
Serum A		
Control precipitate after dialysis	156	156
Precipitate treated with hapten and dialyzed	101	38
% recovered	65	24
Serum B		
Control precipitate after dialysis	44*	59*
Precipitate treated with hapten and dialyzed	24	20
% recovered	55	34

*These figures were obtained on two different occasions.

It is evident that treatment of the antigen–antibody complexes with hapten resulted in lowering the amount of precipitable antibody in spite of extensive dialysis. This effect was more pronounced when *p*-iodobenzene arsonic acid was used as the inhibiting hapten, indicating that the latter hapten was more strongly bound at the antibody site than *p*-arsanilic acid. Independent evidence for this stronger binding of *p*-iodobenzene arsonic acid was also obtained when antibodies were eluted with this hapten from the immunosorbent, i.e. precipitable antibody was recovered with a purity of only about 30% as compared with a purity of 60% obtained on elution with *p*-arsanilic acid.

In a few experiments in which antibodies were dissociated off by lowering the pH to 3.2, the yields were of the order of 40–50% and the purity ranged from 60–70%. However, in some cases, as indicated in Fig. 1, the precipitin curves exhibited large trailing in antigen excess.

[4]This compound was used instead of an appropriately, radioactively labelled *p*-arsanilic acid, because of the low yield obtained in the preparation of the latter compound and because of the prohibitive cost of the labelled starting materials.

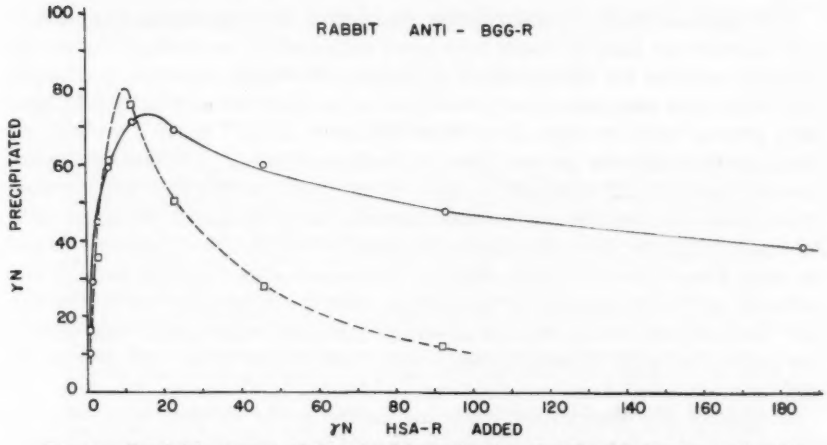

FIG. 1. Precipitin curves obtained with antibodies eluted with hapten and acid: ○ acid eluate, □ hapten eluate.

Discussion

The present investigation represents an attempt to modify and improve the method of Landsteiner and van der Scheer (1) for the isolation of antihapten antibodies with stroma–hapten conjugates. As stated previously, the purity of antibody preparations obtained by these workers, on dissociation with acid, was of the order of 50%. Moreover, Eisen and Karush (2), using the same type of immunosorbent and elution procedure, claimed a purity of 90% for their antibody preparations. However, it appears that their calculations were based on the amount of material removed from the eluates on readsorption with stroma–hapten conjugates and not on the amount of precipitable material determined directly in the eluates. Making similar assumptions, purities of the order of 92% could have been also claimed in this study. On the other hand, as shown in Table II, the antibody content of the eluates computed from precipitin curves amounted to 50–65%. The discrepancy between these data arrived at by the two different methods of calculation would indicate that some antibody sites remained blocked by the eluting hapten even after prolonged dialysis. This interpretation is further supported by the hapten-inhibition experiments, in as much as the amount of specific precipitate after dissolution with hapten and after subsequent dialysis decreased to a value of about 60%. These observations are in agreement with the concept of heterogeneity of antibodies (18), suggesting that the hapten could be removed by dialysis only from antibody sites with low affinities, and would rule out the possibility that the eluates contained significant amounts of non-antibody proteins. The absorption experiments done with normal rabbit γ-globulins demonstrate further that there was no uptake of heterologous γ-globulins by the immunosorbent. Similarly no albumin was removed, as shown by the electrophoresis experiments.

It is obvious that an unequivocal proof that all the eluting hapten was not removed by dialysis could have been obtained by analyzing directly the dialyzed solution for the presence of hapten. However, since the *p*-arsanilic acid which was assumed to have remained in the dialyzed solution would have been present only in trace amounts of the order of 10^{-8} to 10^{-9} mole/ml no attempt was made for its quantitative analysis. Instead, I^{131}-labelled *p*-iodobenzene arsonic acid was used in spite of its known ability to combine more strongly than *p*-arsanilic acid with antibodies directed against the latter (19). As suspected, after dialysis, about 1 molecule of this hapten remained bound for each 2 molecules of eluted protein. Additional evidence that some of the antibody sites were blocked by the eluting hapten was brought out by the fact that the apparent purity of the antibody preparations was lower when elution was performed with *p*-iodobenzene arsonic acid rather than with *p*-arsanilic acid.

In spite of the observation made in another study in this laboratory (9) that elution of antiprotein antibodies with acid from polystyrene–antigen complexes resulted in modification of the eluted antibodies, this method was also resorted to in the present investigation. However, the purity of the antibodies eluted with acid was not higher than that obtained by elution with hapten. Moreover, from the respective precipitin curves (Fig. 1) it was evident that the antibody eluted with acid exhibited a higher degree of heterogeneity.

In conclusion, the weight of evidence obtained in the present investigation would suggest that the proteins eluted with hapten from the stroma–hapten conjugates are primarily antibody γ-globulins and that the apparent lack of purity of the antibody preparations was due to the decrease in precipitability of these antibodies caused by hapten molecules tenaciously bound to some antibody sites.

References

1. K. LANDSTEINER and J. VAN DER SCHEER. J. Exptl. Med. **63**, 325 (1936).
2. N. EISEN and F. KARUSH. J. Am. Chem. Soc. **71**, 363 (1949).
3. D. H. CAMPBELL, R. H. BLAKER, and A. B. PARCEE. J. Am. Chem. Soc. **70**, 2496 (1948).
4. S. I. EPSTEIN, P. DOTY, and W. C. BOYD. J. Am. Chem. Soc. **78**, 3306 (1956).
5. F. KARUSH and R. MARKS. J. Immunol. **78**, 296 (1957).
6. D. H. CAMPBELL, E. LUESCHER, and L. S. LERMAN. Proc. Natl. Acad. Sci. U.S., **37**, 575 (1951).
7. H. SCHNEIDER and A. H. SEHON. Unpublished data.
8. H. C. ISLIKER. Ann. N.Y. Acad. Sci. **57**, 225 (1953).
9. L. GYENES and A. H. SEHON. Can. J. Biochem. and Physiol. **38**, 1235(1960).
10. Y. YAGI, K. ENGEL, and D. PRESSMAN. J. Immunol. **85**, 375 (1960).
11. M. HEIDELBERGER and F. E. KENDALL. J. Exptl. Med. **50**, 809 (1929).
12. H. A. MCKENZIE and H. S. WALLACE. Australian J. Chem. **7**, 551 (1954).
13. F. G. MANN and B. C. SAUNDERS. Practical organic chemistry. Longmans, Green and Co., London. 1947. p. 128.
14. W. C. WOLFE, J. W. WOOD, L. W. KLIPP, T. D. FONTAINE, and J. W. MITCHELL. J. Org. Chem. **14**, 900 (1949).
15. H. A. SLOVITER. Science, **110**, 687 (1949).
16. L. G. LONGSWORTH. Anal. Chem. **23**, 346 (1951).
17. J. R. MARRACK, H. HOCH, and R. G. S. JOHNS. Brit. J. Exptl. Pathol. **32**, 212 (1951).
18. D. W. TALMAGE. J. Cellular Comp. Physiol. **50**, Suppl. 1, 229 (1957).
19. D. PRESSMAN. *In* Molecular structure and biological specificity, *edited by* L. Pauling and H. A. Itano, American Institute of Biological Sciences, Washington. 1957.

TERMINAL AMINO ACIDS OF EGG YOLK LIPOPROTEINS[1]

J. M. NEELIN[2] AND W. H. COOK

Abstract

The N-terminal amino acids of the two lipovitellins and lipovitellenin of egg yolk have been determined. α-Lipovitellin and β-lipovitellin were separated by chromatography on hydroxyapatite but the lipovitellenin in the low-density fraction did not yield stable and reproducible chromatographic components. The major N-terminal amino acids recovered as dinitrophenyl derivatives from all three fractions were arginine and lysine. Further purification removed several minor components, but tyrosine and serine remained in vitellin, and alanine and serine in vitellenin. The principal C-terminal amino acid of vitellenin was glutamic acid. Arginine appeared to be the chief N-terminal residue but difficulty in purifying the water-soluble dinitrophenyl-amino acids led to highly variable yields; thus the total calculated amounts of terminal amino acids ranged from 9 to 35 micromoles per gram of protein. At least two polypeptide chains are indicated, with an average molecular weight less than the size of the total protein moiety of the parent lipoprotein. This is consistent with reported physical measurements in formic acid. The three lipoprotein fractions are indistinguishable on the basis of their terminal amino acids.

Introduction

Three lipoproteins have been isolated from the egg yolk of domestic fowl and have been termed lipovitellenin (1, 2), α-lipovitellin, and β-lipovitellin (3). The chromatographically separated lipovitellins, whole low-density fraction (LDF), and lipid-free vitellenin were used in these studies; there appeared to be no advantage for protein studies in preparing lipovitellenin, containing 40% lipid as originally defined (1), by ether extraction of LDF, containing 90% lipid (2). As the gross chemical composition and physical properties of LDF and the two lipovitellins are quite reproducible, they are evidently distinct and separate entities.

The demonstrated differences between the three lipoproteins of egg yolk lie mainly in physical properties (3, 4), affinity for lipid (1, 2, 5), and particle or molecular size (4, 5). In the few reports on the chemical compositions of their protein moieties (1, 6, 7), different workers have not always employed strictly comparable preparations and often the preparative procedures failed to separate the fractions currently recognized. The amino acid compositions of α- and β-vitellin appear to be the same (3) but there may be small differences in the protein phosphorus content of the three lipoproteins (3, 6). As a further step in establishing the chemical character and interrelations of the protein moieties, end-group assays were undertaken on LDF prepared by ultracentrifugation, and on α- and β-lipovitellin separated by chromatography.

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[2]National Research Council Postdoctorate Fellow, 1959.

Preparations and Methods

Egg yolk protein fractions were prepared as previously described (5). Briefly, egg yolk fluid was diluted with one volume of 1 *M* sodium chloride solution and the low-density fraction (LDF) and the high-density fraction (HDF) were separated by centrifugation at 105,000 *g* for 18 hours at 5° C. Chromatography of HDF on hydroxyapatite was performed as described by Bernardi and Cook (3) with stepwise elution of the livetins, β-lipovitellin, and α-lipovitellin, with 0.2 *M*, 0.6 *M*, and 2.0 *M* potassium phosphate (pH 7), respectively. Protein concentration of the eluents was measured by absorption at 280 mμ.

The N-terminal residues were determined by the 1-fluoro-2,4-dinitrobenzene (FDNB) method of Sanger (8). The reaction was usually performed in saturated guanidine hydrochloride (9) but occasionally in two volumes of methanol (10). The dinitrophenyl-protein (DNP-protein) was prepared both before and after extraction of lipid with chloroform–methanol (2:1 by volume). The lipid-free DNP-protein was found easier to manipulate during washing and extraction. The reaction of vitellenin with FDNB was also performed in 0.1 *M* sodium hydroxide (pH 12) without apparent effect on the results. Hydrolysis, ether extraction, and chromatography of the ether-soluble DNP-amino acids followed the conditions described by Smith and Turner (11). One sample of vitellenin was hydrolyzed for 72 hours. Recovery of di-DNP-lysine was determined as suggested by Levy (10). To test for DNP-proline and DNP-glycine, samples of DNP-vitellin and DNP-vitellenin were hydrolyzed in concentrated hydrochloric acid for 4.5 hours at 100° C in sealed bulbs.

After ether extraction the aqueous phase was examined chromatographically (10). As well as the large amount of ϵ-DNP-lysine present, a weaker zone, presumably DNP-arginine, migrated just ahead of its leading edge. Even when this area was excised, eluted with acidified acetone, and rechromatographed, the two derivatives were not cleanly separated. Removal of contaminating salts and amino acids by passage through a talc–celite column (12) of 18-cm length improved the paper chromatographic separation, but the use of talc columns suffered two disadvantages: the recovery of DNP-arginine was reduced, and the purification was less effective with large samples.

Assay for DNP-arginine was also attempted by a paper electrophoretic method based on that of Burley and Horden (14). The residual aqueous phase was brought to pH 8 with sodium bicarbonate and applied as a line on Whatman paper, Grade 3MM. After impregnation with 1% sodium bicarbonate added by pipette to sharpen the zone, the paper was sandwiched between plate glass, pressed under lead, and subjected to electrophoresis for 3 to 6 hours at 12–20 volts per cm of length, 2 to 3 mm per cm of width.

Two modifications of the Sakaguchi reaction were used for detection of arginine derivatives after paper chromatography. In the first, the paper was dipped in 0.1% α-naphthol in ether, and then was sprayed with 5% sodium hypochlorite. The second method used the more refined reagents of Irrevere *et al.* (15), including a stabilizing spray of iodine and potassium iodide.

Attempts to confirm the identification by hydrolysis of the DNP-arginine in saturated barium hydroxide solution (16) were inconclusive. Amino acids before and after hydrolysis were analyzed on a 50-cm column (17) of a Beckman–Spinco Model 120 amino acid analyzer. Residual amino acids and their degradation products, including ornithine, from the prior acid hydrolysis were sufficient to mask the relatively small changes due to the alkaline hydrolysis.

The C-terminal residues were determined by hydrazinolysis in the presence of hydrazine sulphate and redistilled hydrazine as described by Bradbury (18). Amino acid hydrazides were removed by repeated precipitation with benzaldehyde and the free amino acids determined by reaction with FDNB and paper chromatography of their DNP derivatives. Rechromatography of the eluted DNP-amino acids was necessary to complete the separation from the great excess of dinitrophenol and residual DNP-hydrazides.

Ultracentrifugal examinations of the yolk solutions and their fractions were made in a Spinco Model E ultracentrifuge at ca. 250,000 *g* at 20° C. Fractions obtained by chromatography on hydroxyapatite were examined in the phosphate buffer in which they were removed from the column.

Results

Chromatography

Because of the apparent physical polydispersity of LDF (4) and the promising fractionation indicated by preliminary chromatograms on hydroxyapatite (3), this procedure was investigated before end-group assays were undertaken. Stepwise elution with solutions of increasing phosphate concentration was followed by measurement of turbidity (optical density at 500 mμ) in a Coleman Model 6A colorimeter. Quantitative recoveries of peaks are not quoted, as degree of turbidity may be susceptible to changes in ionic environment. The results presented in Fig. 1A show that LDF was separated into two major peaks and several minor peaks and shoulders. However, the proportions of these varied with the ratio of sample to adsorbent, even when the column was far from saturated; peak 3 was relatively greater when the proportion of sample was increased. When the flotation properties of the two major chromatographic peaks were compared with that of the original LDF, the polydispersity was still evident in both (Fig. 1A). Rechromatography of three chromatographic fractions (including the major peaks), dialyzed against 0.05 *M* phosphate buffer, yielded only material eluting as peak 3, i.e., at the highest eluent concentration (Figs. 1B to 1D).

End-group Assays

The main N-terminal residues of vitellenin were lysine and arginine, tentatively identified by chromatography. The identification was strengthened by paper electrophoresis of the water-soluble DNP-derivatives. Besides the cationic DNP-arginine, six anionic zones included ε-DNP-lysine and fast minor zones, presumably degradation products and peptides. With large samples

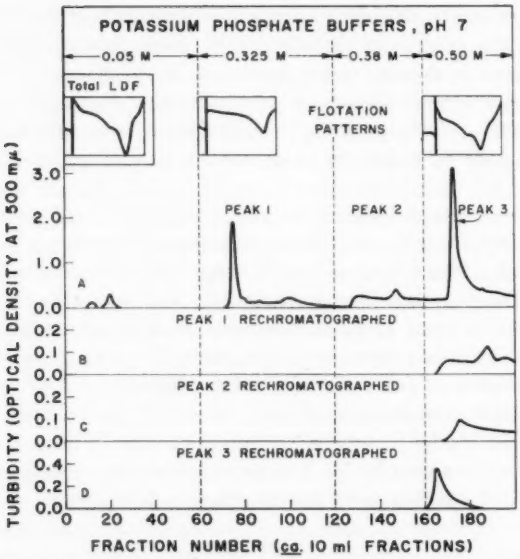

FIG. 1. Chromatography of low-density fraction (LDF) of egg yolk on hydroxyapatite columns.
(A) Whole LDF. Inset are ultracentrifuge patterns of LDF before chromatography and of the major chromatographic fractions, peak 1 and peak 3. (B) Peak 1 of (A), rechromatographed. (C) Peak 2 of (A), rechromatographed. (D) Peak 3 of (A), rechromatographed.

both major zones streaked back to the origin, even after a second electrophoresis of each excised and eluted zone. The eluted DNP-arginine remained cationic upon repeated electrophoresis and was chromatographically distinct from dinitroaniline. Internal standards of added DNP-arginine (19) could not be distinguished from the N-terminal component in these analyses.

Sakaguchi reactions indicated substituted guanidine in the presumptive DNP-arginine zone. With the method using concentrated sodium hypochlorite, both standard DNP-arginine and the N-terminal derivative turned a stable orange-brown, along with a hitherto colorless region, presumably free arginine, extending from ε-DNP-lysine to the origin. The method of Irrevere *et al.* (15) gave a typical pink-brown DNP-arginine color which faded rapidly while a standard sample was stable. After purification by repeated paper electrophoresis and paper chromatography, a stable color was obtained. Free arginine was readily distinguished at the origin of the chromatogram. Sakaguchi tests of the electrophoretic zone were inconclusive because of the near coincidence with free arginine.

The recovery of arginine as the DNP-derivative was highly variable, from 4 to 30 μmoles per gram of protein, by both chromatographic and electrophoretic methods of analysis. This variation was independent of the lipoprotein preparation but appeared related to the sample size, as it was observed with

different aliquots of the same hydrolyzate. It is suspected that contaminating dinitrophenylated material, possibly ε-DNP-lysine or degradation product, had considerable affinity for the DNP-arginine and was particularly poorly resolved from it in large samples. The most consistent recovery, 1.1 to 1.5 μmoles from 80 to 100 mg of vitellin or vitellenin, was used for the comparisons in Table I; the relative values for the three proteins are more reliable than absolute amounts, since comparable samples of the three proteins were used. The ether-soluble DNP-amino acids were readily determined and quantitatively reproducible. The recovery of added di-DNP-lysine from LDF was 58%, and this factor was used to correct for losses of this amino acid; that of DNP-arginine could not be determined because of the quantitative limitations mentioned. No evidence of DNP-proline or its derivatives was found after sealed bulb hydrolysis and all other residues were less than after reflux hydrolysis. The possibility of resistant peptides was examined by continuing reflux hydrolysis for 72 hours. Only the DNP-derivatives of arginine, alanine, and glutamic/aspartic acids were recovered and these in greatly reduced amounts. Reaction with FDNB in two volumes of methanol rather than in saturated guanidine hydrochloride did not alter the results. The minimum and maximum yields of DNP-amino acids, 9 and 35 μmoles per gram of protein, would indicate number average "molecular" weights (M_n) of ca. 3 to 11×10^4; the most consistently obtained yield was 19 μmoles per gram (Table I) or 1 mole per 5×10^4 grams of protein.

TABLE I

Terminal amino acids of vitellenin and vitellin
(μmoles amino acid/g protein)

Amino acids	Vitellenin		α-Vitellin	β-Vitellin
	N-terminal*	C-terminal	N-terminal*	N-terminal*
Arg	(14)†	0.0	(13)†	(13)†
Lys	2.9†	2.7	3.1†	1.9†
Tyr	0.0	0.0	0.7	0.9
Ser	0.5	0.5	0.8	0.2
Ala	0.9	5.8	0.0	Trace
Glu	0.3	18.2	0.0	0.2
Asp		2.7		
Val, Thr, His	0.2	0.0	0.0	0.2
Leu	0.0	4.0	0.0	0.0
Gly	0.0	5.5	0.0	0.0
Total	(19)	39	(18)	(16)

*Water-soluble DNP-amino acids separated by paper electrophoresis, ether-soluble DNP-amino acids by paper chromatography.
†Lysine only is corrected for losses (58% recovery), since recovery of arginine was highly variable (4 to 30 μmoles/g vitellenin). The tabulated values were averages from 80 to 100 mg of each protein.

The apparent C-terminal residues of vitellenin are given in Table I. Glutamic acid was the main residue, representing about half the total yield. While six minor components made up the remainder, it was not possible to distinguish minor terminal residues from the by-products of hydrolysis of amino acid

hydrazides. The uncorrected yield of C-terminal glutamic acid is comparable with the uncorrected yield of N-terminal arginine.

The main N-terminal residues of both α- and β-lipovitellin were again arginine and lysine, with traces of others (Table I). The same difficulty in separating the water-soluble DNP-derivatives was experienced but duplicate analyses with comparable amounts of sample gave average yields in agreement with that from lipovitellenin. In these proteins tyrosine was consistently present and the absence of this amino acid in vitellenin is the only noteworthy difference between the two lipoproteins. However, the small amount present and its variability in different preparations indicate that it probably arises from a contaminating protein. There was no evidence of DNP-proline after sealed bulb hydrolysis. The recovery of DNP-arginine after reflux hydrolysis of β-lipovitellin was apparently more than 90%, but the difficulty in removing contaminants makes such quantitative determinations doubtful. However, DNP-arginine is known to be relatively stable to these treatments, since Burley and Horden (14) found 70% recovery after hydrolysis in the presence of wool proteins and electrophoretic separation. The yields of N-terminal residues, while subject to the uncertainties mentioned above, indicate that the two vitellins have the same number average molecular weight as vitellenin.

The occurrence of several end groups in these preparations of vitellin and vitellenin suggests heterogeneity, impurities, or a lability of these molecules. These alternative possibilities were investigated by examination of the ether-soluble DNP-amino acids of more exhaustively purified LDF, LDF fractions, LDF in strong solvents, and degraded lipovitellin (Table II). The ether-soluble DNP-residues provided a more sensitive criterion because they are amenable to quantitative analysis. Table II summarizes averages of at least duplicate analyses of two or more independent preparations of LDF. The proportion of any ether-soluble DNP-amino acid usually varied less than 10% in different preparations given the same treatment.

After repeated washings by suspension in 1 *M* sodium chloride and centrifugation, four of the five trace amino acids of LDF (valine, threonine, leucine, histidine) were absent. Chromatography on hydroxyapatite accomplished a similar purification, but had no operational advantage over the washing procedure. Chromatography also reduced contamination of α- and β-lipovitellin but did not remove tyrosine completely.

As lipid-free vitellenin is insoluble in neutral solution, 88% formic acid and 0.1 *M* sodium hydroxide have been used as solvents for physical studies (4). If degradation occurs in these solvents, it should change the N-terminal amino acids quantitatively or qualitatively. Solutions in 0.1 *M* sodium hydroxide (pH 12.0) were allowed to stand overnight at 5° C and the FDNB reaction performed at this pH. Solutions in 88% formic acid were stored at 5° C overnight, the formic acid removed in a rotary evaporator, and the FDNB reaction performed in the usual way. The results in Table II show that over this period neither solvent caused end-group changes which exceeded differences between

preparations, except for the somewhat higher content of N-terminal valine in vitellenin in sodium hydroxide; this may be indicative of incipient degradation.

The two fractions obtained from chromatographic separation of LDF on hydroxyapatite yielded the same ether-soluble DNP-amino acids in similar proportions (Table II). This, coupled with evidence from ultracentrifugation and chromatography (see above), indicates the probable identity of these fractions.

TABLE II

Effect of various treatments on the N-terminal residues of vitellenin and vitellin

Treatment	N-terminal amino acids as percentage of total ether-soluble DNP-amino acids recovered*					
	Lys	Ser	Ala	Asp, Glu	Val	Thr, Leu, His
				Vitellenin		
Untreated†	72	9	5	4	7	3
Washed three times	78	10	6	6	0	0
After lipid ext.†	51	18	19	7	3	2
In 88% formic acid	63	25	8	Trace	2	2
In 0.1 *M* NaOH	49	19	10	4	12	6
Chromatographed						
Peak 1 + 2 (Fig. 1)	84	9	Trace	7	0	0
Peak 3 (Fig. 1)	82	11	4	3	0	0

	Lys	Ser	Ala	Asp, Glu, His	Val	Tyr	Thr
				Vitellin			
Untreated α- + β-	43	14	11	8	Trace	24	Trace
Degraded α-	Trace	4	2	1	72	0	21
Degraded β-.	1	2	3	<1	55	0	38

*All ether-soluble DNP-amino acids represent less than half of total N-terminal amino acids.
†Includes FDNB reactions both in saturated guanidine hydrochloride and in two volumes of methanol.

Previous experience had indicated that microbiological degradation during preparation at 5° C was most likely to occur during chromatography on hydroxyapatite columns. The effect of such degradation was investigated by comparing unfractionated lipovitellin with the α- and β-fractions obtained from hydroxyapatite columns that had become contaminated (Table II). In both vitellins this treatment generated two new N-terminal amino acids, valine and threonine, which were present in only trace amounts in the untreated material or in native fractions. On the other hand, less DNP-lysine was recovered from the degraded proteins. Evidently the lysine, serine, and tyrosine found in most preparations were not generated by incipient biological decomposition, as the content of these amino acids was diminished rather than enhanced by this treatment. If the uncontrolled microbiological proteolysis is considered as a coarse "fingerprinting" analysis, the results obtained with α- and β-lipovitellin emphasize the similarity of the main peptide chains.

Discussion

The low-density fraction of egg yolk is evidently polydisperse (4) but attempts to fractionate it by chromatography on hydroxyapatite columns, as suggested by Bernardi and Cook (3), have been unsuccessful. The two major fractions separated were not reproducible on rechromatography and may have depended on metastable phosphate–lipoprotein complexes. A somewhat similar behavior has been reported for nucleic acid on calcium phosphate columns (20).

The major N-terminal amino acids of the two vitellins and vitellenin were found to be the same in kind and content. Arginine appears to be the major N-terminal residue, amounting to at least half of the recovered DNP-derivatives. The qualitative recognition of DNP-arginine was based on the following facts: First, two chromatographic zones were observed in the ε-DNP-lysine/DNP-arginine region, with improved separation by prior chromatography on paper or on a talc column. Second, the unknown component was cationic in 1% sodium bicarbonate, even after repeated paper electrophoresis, while all other components were anionic. Third, this DNP-derivative was separated from ε-DNP-lysine by paper electrophoresis in 1 *M* ammonium hydroxide (13). In all these analyses the presumptive DNP-arginine could not be distinguished from internal or lateral standards of known DNP-arginine. Fourth, the chromatographically separated zone was Sakaguchi positive, but distinct from free arginine, and ninhydrin-negative if adequately purified (13).

Inconsistencies included the failure of the N-terminal derivative to separate discretely from ε-DNP-lysine, especially in large samples, and the instability of its Sakaguchi color. It is believed that these features were due to the 30-fold excess of ε-DNP-lysine and to possible concomitants, since purification gave more definitive results. Unfortunately, the methods of purification on paper and on talc columns all appear to be less effective as the sample size is increased This prevented unequivocal identification by chemical means.

Valine, threonine, leucine, and histidine, four of the N-terminal amino acids found in trace amounts, can be removed by washing or by chromatography, and must arise from contaminating protein. Alanine is also reduced by chromatography. However, lysine and serine (and tyrosine in the vitellins) were consistently present, and there is no evidence that their presence can be attributed to degradation of the lipoprotein during preparation. Identical results from reaction in alcohol and in presence of guanidine hydrochloride suggest that bound peptides or amino acids do not contribute to these components. They must indicate either a basic heterogeneity of the protein moieties or the presence of trace proteins interacting strongly and perhaps specifically with the lipoproteins.

With the information now available, some modification of previous conclusions in papers from these laboratories is required. In an investigation confined to comparison of soluble and insoluble lipovitellenin fractions, lysine was reported as the major ether-soluble DNP-amino acid of both (11). While this

has been confirmed, the ether-soluble DNP-amino acids constitute no more than about half of the total. It was also reported (5) that γ-livetin as well as phosvitin were probable components of certain lipovitellin preparations. Since it is now known that alanine is the main N-terminal amino acid of both these proteins (21), neither could be significant contaminants of present preparations.

While the identification of N-terminal arginine in these lipoproteins is not unequivocal, there is evidence of a water-soluble DNP-derivative with the properties of arginine or a closely related compound. Quantitative analyses of water-soluble DNP-amino acids must be considered uncertain, at least from some proteins, and we concur with the comments of Biserte *et al.* (12) on the difficulties involved. Nevertheless, useful comparisons can be made; when the three lipoproteins were reacted in parallel under identical conditions with similar sample sizes, their major end groups did not differ significantly.

The parent lipoproteins differ mainly in their lipid content and molecular weight, from which the respective sizes of the protein moieties, vitellenin and vitellin, were computed to be 1.6×10^5, and 3.2×10^5, in terms of molecular weight. This difference in size may not be a distinguishing feature, since both lipovitellins dissociate into two subunits in solvents at pH 9.0 or higher (3); vitellenin may be a vitellin subunit stabilized against association at neutrality by interaction with additional lipid. Even with the minimum observed yield of DNP-arginine, the total yield was at least 1 mole per 10^5 grams of protein. The yield of C-terminal glutamic acid from vitellenin, all estimates of N-terminal groups, and physical measurements in strong solvents (4), all agree that the average size of the polypeptide chains is less than the computed protein moiety. Breakage of peptide bonds was not a factor in the physical measurements (4), since no new end groups were generated by exposure to formic acid during the period of the experiment. Evidently, several polypeptide chains must compose the protein moiety of each egg yolk lipoprotein.

Acknowledgments

The authors are indebted to Dr. R. W. Burley for suggesting the paper electrophoretic method of separating the basic DNP-amino acids, and for advice about its application and interpretation. Dr. W. G. Martin provided generous co-operation in many aspects of the problem, notably in the purification on talc columns. J. Labelle performed valuable analyses on a Beckman–Spinco amino acid analyzer.

References

1. H. L. Fevold and A. Lausten. Arch. Biochem. **11**, 1 (1946).
2. K. J. Turner and W. H. Cook. Can. J. Biochem. and Physiol. **36**, 937 (1958).
3. G. Bernardi and W. H. Cook. Biochim. Biophys. Acta, **44**, 96 (1960).
4. W. G. Martin, K. J. Turner, and W. H. Cook. Can. J. Biochem. and Physiol. **37**, 1197 (1959).
5. F. J. Joubert and W. H. Cook. Can. J. Biochem. and Physiol. **36**, 389 (1958).
6. R. C. Warner. The proteins. Vol. II, Part A. *Edited by* H. Neurath and K. Bailey. 1954. p. 474.

7. J. C. LEWIS, N. S. SNELL, D. J. HIRSCHMANN, and H. FRAENKEL-CONRAT. J. Biol. Chem. **186**, 23 (1950).
8. F. SANGER. Biochem. J. **39**, 507 (1945).
9. D. M. P. PHILLIPS. Biochem. J. **68**, 35 (1958).
10. H. FRAENKEL-CONRAT, J. I. HARRIS, and A. L. LEVY. Methods of Biochem. Anal. **2**, 359 (1955).
11. D. B. SMITH and K. J. TURNER. Can. J. Biochem. and Physiol. **36**, 951 (1958).
12. G. BISERTE, J. W. HOLLEMAN, J. HOLLEMAN-DEHOVE, and P. SAUTIERE. Chromatog. Reviews, **2**, 59 (1960).
13. W. G. MARTIN. Personal communication.
14. R. W. BURLEY and F. W. A. HORDEN. Textile Research J. In press.
15. F. IRREVERE, D. KOMINZ, and A. HAYDEN. Personal communication.
16. G. L. MILLS. Nature, **165**, 403 (1950).
17. D. H. SPACKMAN, W. H. STEIN, and S. MOORE. Anal. Chem. **30**, 1190 (1958).
18. J. H. BRADBURY. Biochem. J. **68**, 482 (1958).
19. R. R. PORTER and F. SANGER. Biochem. J. **42**, 287 (1948).
20. R. K. MAIN, M. J. WILKINS, and L. J. COLE. J. Am. Chem. Soc. **81**, 6490 (1959).
21. J. M. NEELIN and W. H. COOK. In preparation.

EFFECT OF CHANGES OF AMBIENT TEMPERATURE ON SPONTANEOUS ACTIVITY OF HYPOTHYROID RATS[1]

MELVIN J. FREGLY

Abstract

Administration of the antithyroid drug, propylthiouracil (PTU), to male rats during 4 weeks did not reduce spontaneous running activity in air at 25° C. When room temperature was lowered to 8° C for 4 days, PTU-treated rats increased their activity level during the 1st day of exposure but decreased it thereafter. Control rats responded similarly on the first day but continued to increase activity during each subsequent day in cold. In other experiments PTU-treated and control rats were lightly restrained and exposed *acutely* to air at 5° C in individual cages. A sensitive transducer converted movement of the cage to electrical impulses which were recorded on a polygraph. Colonic temperature of each rat was recorded simultaneously by a potentiometer. Mean colonic temperature of control rats decreased 2 to 3° C during the first 2 hours of cold exposure but was maintained at this subnormal level for an additional 2.5 hours by adjustment of activity. Activity increased immediately with cold exposure and became maximal when colonic temperature fell 0.5 to 1° C. Mean colonic temperature of PTU-treated rats continued to fall throughout the cold exposure and decreased approximately 6° C in 3 hours. These rats also responded to cold initially with an increase in activity which became maximal when colonic temperature fell 3 to 4° C. However, this maximal effort occurred too late to prevent further fall of colonic temperature. The results suggest that regulation of body temperature in cold air by adjustment of activity level is an important aspect of the total regulatory mechanism and that hypothyroidism alters the relationship between colonic temperature and activity level.

Recent studies have shown that hypothyroid rats cool faster than control rats when restrained and subjected to air at 5° C (1). At this same air temperature, unrestrained, hypothyroid rats given adequate food and water may survive for a few days but usually succumb to cold within 6 to 8 days, while unrestrained, control rats survive for many months (2, 3). This difference in survival has stimulated a number of investigations designed to help elucidate the mechanism(s) destroyed or diminished in intensity as a result of loss of thyroid function. Metabolic rate was one of the first parameters studied and compared. Although the "basal" metabolic rate of hypothyroid rats is lower than that of controls, total metabolic rate observed after these rats are subjected to cold air increases to a level near that of controls (4, 5). Increased muscular activity and shivering probably account for the increased metabolic rate under these conditions (5). Why then does the hypothyroid rat fail to survive exposure to cold air? Does muscular fatigue occur more quickly in the hypothyroid rat, predisposing it to the effects of cold? Does the increased running response to cold observed in normal rats (6, 7) disappear after either reduction or loss of thyroid activity? With respect to spontaneous running

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activity of hypothyroid rats at *normal* room temperatures, early studies have yielded conflicting results. Reports of a decrease (8, 9) and no change (10, 11, 12) have been made. Nothing is known of the running response of hypothyroid rats to cold air. An initial objective of these experiments was to compare running activities of control and hypothyroid rats kept in air at 25° C and, later, to determine the effect of both short-term and acute changes in ambient temperature on activity level.

Methods and Results

Four separate experiments were performed. Male rats of the Wistar strain were used in experiment 1 while male rats of the Holtzman strain were used in experiments 2 to 4 inclusive. The first two experiments were designed: (*a*) to test the effect of hypothyroidism, produced by means of the drug propylthiouracil, on the spontaneous running activity of rats kept in air at 25° C (experiment 1); and (*b*) to test the effect of changes in ambient temperature on spontaneous running activities of control and propylthiouracil-treated rats (experiment 2). The results of these two experiments, in which wheel-type activity cages were used, will be described first; the results of the last two experiments, which used a different method for assessment of activity, will be described later.

The activity cages used in the first two experiments were standard wheel-type cages available commercially.* The wheel diameter was 37.5 cm and the width was 11.5 cm. The number of revolutions turned by the wheel in either direction was counted on a Veeder-type counter. Activity was measured daily at 9 A.M. All rats were given, ad libitum, ground Purina laboratory chow to eat and tap water to drink. The experimental rats were given propylthiouracil† (PTU) mixed thoroughly into food at a level of 0.1% by weight. All experiments were performed in a windowless, thermoregulated room illuminated from 8 A.M. to 6 P.M. The room temperatures varied with each particular experiment and will be given as each experiment is described. Statistical comparison of the data was made by means of the "*t*" test for the 95% confidence limit (13).

Experiment 1: Effect of Propylthiouracil Treatment on Spontaneous Running Activity in Air at 25° C

Two groups of five male rats each weighing 220 to 250 g and approximately 9 weeks old were placed individually in activity cages. The rats were allowed 3 days to accustom themselves to the cages before daily activity measurement began. PTU was given to the second group at the end of the 3rd week in activity cages. The treatment continued for 4 weeks during which activity was measured daily.

The results of the experiment are shown in Fig. 1. Activity of treated rats was higher initially than controls although the difference between activities was not statistically significant. This difference was maintained throughout

*George H. Wahman Co., Baltimore, Md.
†6-*n*-Propyl-2-thiouracil, Nutritional Biochemicals Corp., Cleveland, Ohio.

the experiment and became significant ($P < 0.01$) during the last 2 weeks of the experiment. The increase in running activity to a maximum within 3 weeks after introduction to activity cages and its subsequent decline have been described by others (14). Propylthiouracil treatment did not influence this characteristic activity pattern.

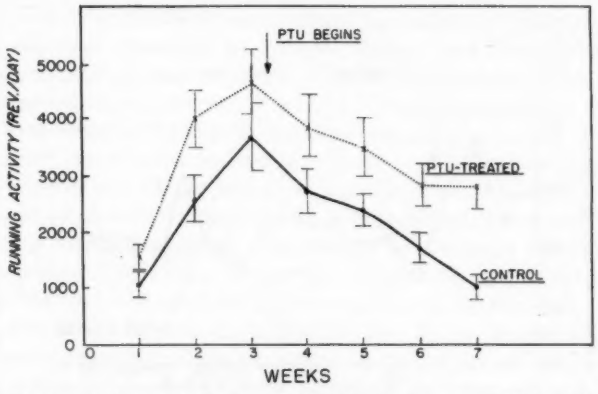

FIG. 1. Mean running activity (revolutions/day) of male PTU-treated (×) and control (●) rats during each week of the experiments; ±1 standard error is set off at each mean. PTU treatment began at the end of the 3rd week of the experiment.

Experiment 2: Effect of Propylthiouracil Treatment on Spontaneous Running Activity at Different Environmental Temperatures

Two groups of five male rats each weighing 350 to 370 g were used. The rats were placed in activity cages and given 10 days to accustom themselves to their new environment. Tap water and ground Purina chow were given ad libitum. Room temperature was maintained at 25±1° C. Activity was then measured daily for 3 weeks after which the rats in group 2 were given chow containing PTU. The experiment continued for 6 more weeks. In the middle of the 9th week, room temperature was lowered to 20° C for 4 days. Thereafter, for 4-day intervals, the room temperature was 25, 15, 8, and 25° C. Relative humidities measured by a sling psychrometer varied from 70 to 80% at 25° C and from 80 to 90% at 8° C.

Figure 2 shows graphically the results of this experiment. Again it was observed that administration of PTU did not significantly affect running activity during the period at 25, 20, or 15° C. When ambient temperature was lowered to 8° C, however, running activity of control rats increased significantly, while that of PTU-treated rats fell significantly. Comparison of the daily activity pattern at this low temperature indicates that PTU-treated rats *increased* activity 12% over previous control level during the *first* day of exposure to 8° C. Control rats increased their activity level 17% during this same time. The major difference between the two groups occurred on subsequent

days when the activity of PTU-treated rats declined to a level approximately half that of their previous control level, while control rats doubled their pre-cold activity level. When ambient temperature was returned to 25° C, activity level of control rats returned to previous level while that of PTU-treated rats fell even lower. This was the case for all individuals tested.

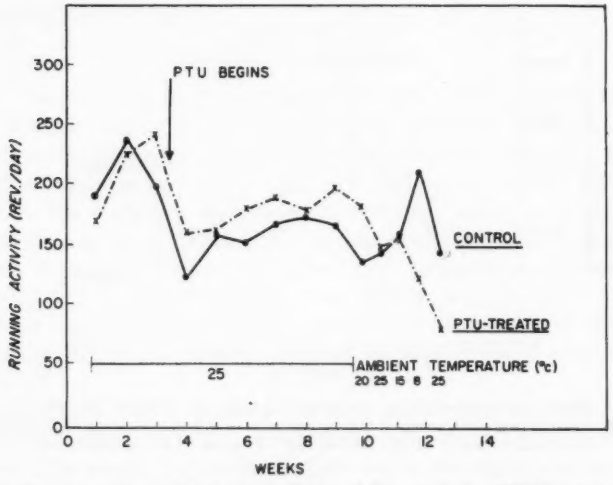

FIG. 2. Mean running activity (revolutions/day) of male PTU-treated (×) and control (●) rats during each week of the experiment. PTU treatment began at the end of the 3rd week of the experiment. Ambient temperatures during the experiment are indicated at bottom of figure.

At the end of the experiment all rats were sacrificed by ether and the thyroid was dissected and weighed on a torsion balance. The thyroid gland of normal rats weighed 5.5 ± 0.5 (S.E.) mg/100 g body weight while that of PTU-treated rats weighed 31.6 ± 6.0 mg/100 g body weight ($P < 0.01$). Hematocrit ratio and hemoglobin concentration (acid hematin method) were also measured on blood removed from the heart at death. The values for normal rats were hematocrit 50.8 ± 1.3 and hemoglobin 16.9 ± 0.5 g% while those of PTU-treated rats were 42.9 ± 1.9 and 13.5 ± 1.6 respectively. Both values were significantly lower for PTU-treated rats ($P < 0.01$). These results, taken together, indicate that the treated rats were hypothyroid since enlarged thyroid gland and anemia accompany hypothyroidism produced by PTU (15).

The difference in absolute activity levels (approximately 10-fold) between the male rats in experiments 1 and 2 (see Figs. 1 and 2) may be the result of strain and age differences. The rats of experiment 1 were Wistar rats; those of experiment 2 were Holtzman. Further, the rats of experiment 1 were younger than those of experiment 2 and younger rats are known to be more active than older rats (14).

Experiment 3: Effect of Acute Exposure to Cold on Activity of PTU-treated Rats

The results of experiment 2 suggested that administration of PTU failed to prevent the characteristic increase in running activity accompanying cold exposure. Activity of PTU-treated rats may have decreased after the first day of cold exposure because body temperature was falling. It therefore seemed worth while to correlate colonic temperature of rats with their moment-by-moment activity when ambient temperature was suddenly lowered.

Cages of the type described by Adolph, Barker, and Hoy (16) were used. These were altered such that the back (tail) end of the cage rested on a knife edge which served as the fulcrum of a lever. A spring supported the front of the cage. Dimensions of the cage were 33 cm long and 9 cm wide. Beneath the cage at the front (head) end was attached the core of a Schaevitz linear-variable differential transformer (Type 033 SL). Movement of the core of the transformer induced an electrical current which was amplified and recorded on paper by means of a Grass polygraph. The weight of the cage provided enough damping to prevent oscillation. Movement of each cage, with rat inside, was calibrated at the beginning and end of each experiment by placing a 50-g weight at the end of the cage farthest from the fulcrum. Each channel was adjusted to give full-scale deflection of the ink writer on the polygraph when the weight was added. Colonic temperature was measured by means of a copper–constantan thermocouple inserted 5 cm into the colon of the rat and held in place by means of adhesive tape which bound it to the tail. The thermocouple was led off to a potentiometer which recorded colonic temperature at 1-minute intervals. Thus, activity level and colonic temperature could be measured simultaneously for each rat. Two rats could be run at a time in these experiments, which were conducted with the rats in a thermoregulated box maintained at $25 \pm 1°$ C during the initial control period and lowered to $5 \pm 1°$ C during the experimental period. Approximately 40 minutes was required to lower box temperature to 5° C. Activity level was assessed by counting the movements of each cage per minute without attempting to integrate the area under the activity curve (total work). The sensitivity of this system was greater than that described by Bartlett *et al.* (17) but probably did not measure non-visible shivering.

Results of these experiments are shown in Fig. 3, in which colonic temperature and activity units are plotted against time. Each point represents an average of either colonic temperatures or activity units at the same 5-minute period of the experiment for six control (Part A) and six PTU-treated (Part B) rats. Control rats showed an over-all increase in activity during cold which amounted to approximately twice that observed before cold exposure. As soon as ambient temperature was lowered, activity level increased. The activity was maximal between 37.0 and 37.5° C colonic temperature (Fig. 4A). Both activity level and colonic temperature fell thereafter. The lowest level of colonic temperature reached by control rats was 36° C. This temperature level seemed to be maintained by bursts of activity when colonic temperature began to fall.

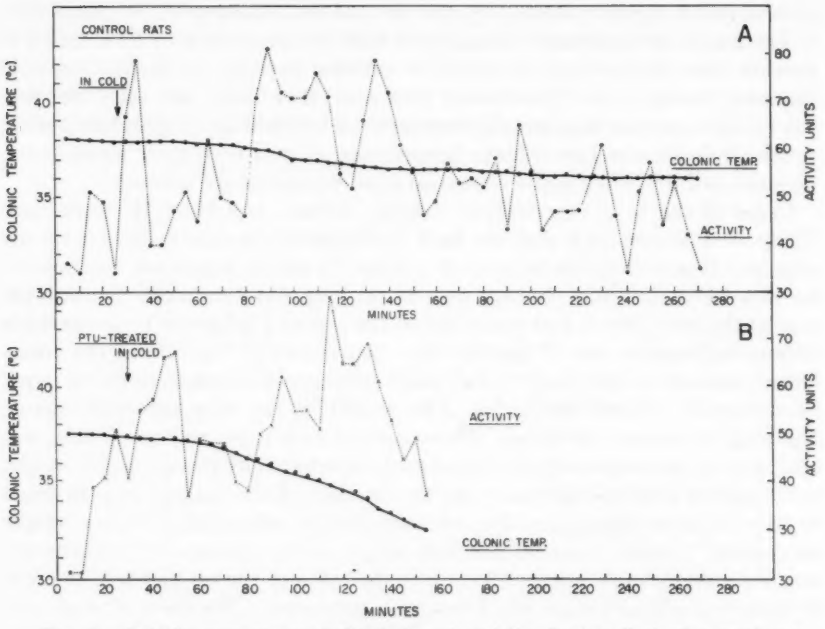

FIG. 3. Colonic temperature (solid line) and activity (broken line) of control rats (Part A) and PTU-treated rats (Part B) during acute exposure to air at 5° C. Activity (arbitrary units) and colonic temperature (°C) are the means of simultaneous measurements taken during each 5-minute period of experiment.

Rise of temperature above 36° C was then followed by cessation of activity. Thus, it may be observed from Fig. 4A that many different activity levels were recorded at colonic temperatures in the vicinity of 36° C. In the case of PTU-treated rats, an over-all increase in activity during cold exposure was also observed. Activity levels during cold exposure were also double that observed prior to cold (Fig. 3B). The absolute levels of activity attained were the same for both PTU-treated and control rats. Activity of PTU-treated rats also increased as soon as box temperature was lowered. This increased activity level was maintained for approximately 20 minutes and then fell to pre-cold exposure levels (Fig. 4B). Colonic temperature began to fall sharply. Activity level then increased and became maximal at a colonic temperature of 34.5 to 35.0° C. Thereafter, activity level fell approximately linearly with decreasing colonic temperature until the rats were removed from cold. The major differences in the responses of the two groups were: (*a*) PTU-treated rats manifested a larger fall in colonic temperature upon exposure to air at 5° C and colonic temperature fell at a much faster rate (2.6° C/hour as compared with 0.5° C/hour); (*b*) activity level of PTU-treated rats was maximal at a lower colonic temperature during cold exposure, suggesting that body defenses against cooling (including

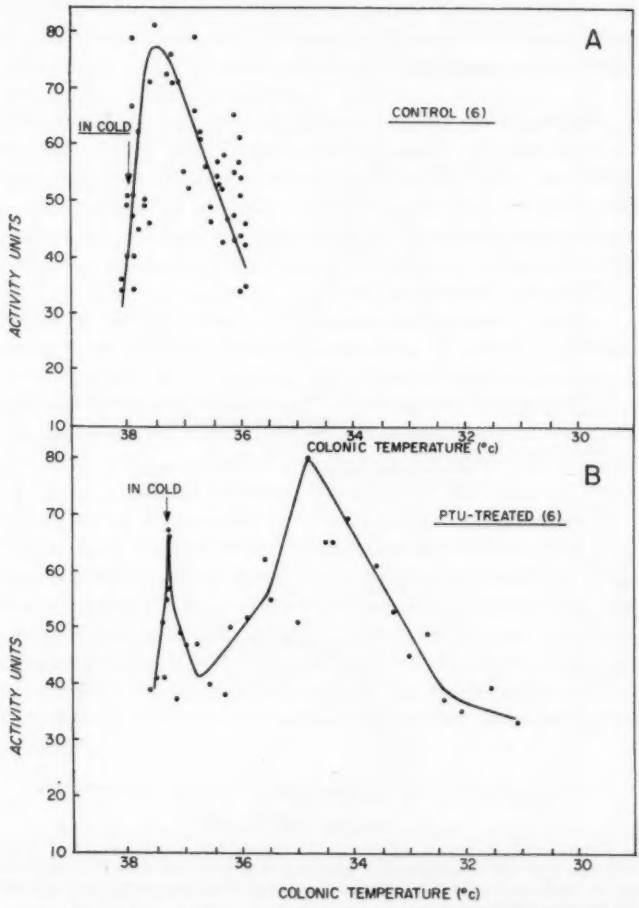

FIG. 4. Relationship between activity level and colonic temperature for six control (Part A) and six PTU-treated rats (Part B). Arrow indicates where exposure to air at 5° C began. See text for further explanation.

activity) began too late to afford adequate protection. The maximal activity levels achieved by both groups were similar. Once activity started in PTU-treated rats, it could achieve the same magnitude (in terms of movement/unit time) as achieved by untreated controls. Whatever the mechanism regulating activity in the cold, it seems to be summoned to action too late in the process of cooling to be beneficial to the PTU-treated rat. It appears that control rats do not regulate activity to maintain their usual colonic temperature (38° C) during cold exposure. Instead, colonic temperature is maintained about 2 degrees below normal level, at least in part, by adjustment of activity. The

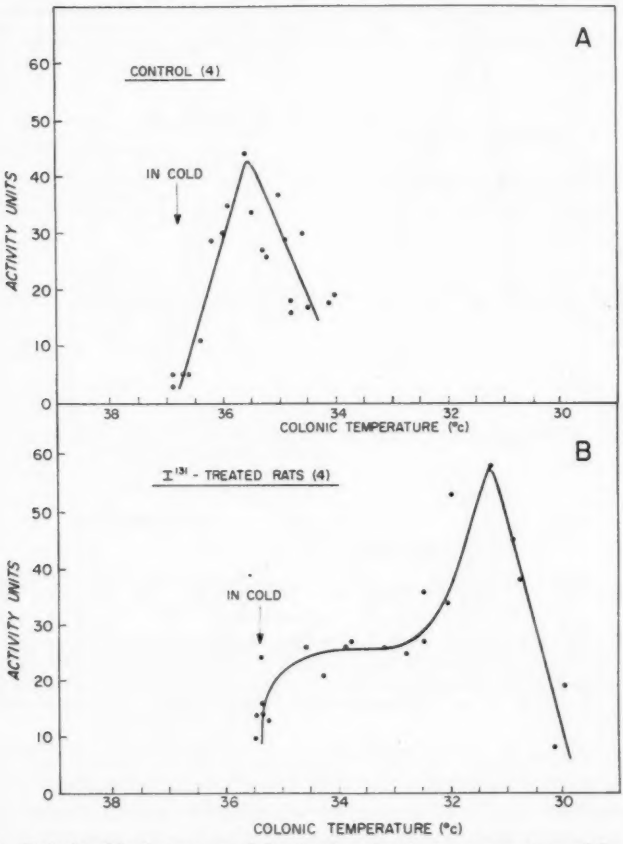

FIG. 5. Relationship between activity level and colonic temperature of four control (Part A) rats and four rats treated with a dose of radioactive iodine large enough to destroy functioning thyroid tissue (Part B). Arrow indicates where exposure to 5° C air began. See text for further explanation.

reason for maintenance of colonic temperature at a relatively hypothermic level is not apparent from these experiments.

Experiment 4: Effect of Acute Exposure to Cold on Activity of Rats Thyroidectomized by Radioactive Iodine

Four male Holtzman rats were injected with 850 μc of radioactive iodine (NaI^{131}) 8 weeks prior to the experiment. Four untreated rats were used as controls. The dosage of radioactive iodine used had been shown to destroy the thyroid gland almost completely (18, 19). All rats were subjected to activity tests in cold air exactly as described in experiment 3. A single difference was that the cages used in this experiment had a metal vane attached to the

front end. This vane was free to move in a pot of light mineral oil which acted to damp movements of the cage. This made the system less sensitive than that used in experiment 3 so that total activity levels of rats in this experiment are less. However, the results are similar.

Figure 5A shows the relationship between colonic temperature and activity for control rats. Each point represents the mean activity over a 5-minute period compared with the mean colonic temperature over the same 5-minute period for all control rats. Activity level increased when ambient temperature was lowered. Activity rose to a maximum at a colonic temperature of 35.6° C and decreased as colonic temperature fell. Colonic temperature did not fall below 34° C in these experiments and was maintained somewhat above this temperature even though some of the experiments lasted 7 hours.

Rats treated with radioactive iodine responded to cold in a fashion similar to that of PTU-treated rats. Activity level doubled approximately as colonic temperature fell to levels of about 34.5° C. As colonic temperature fell further, activity increased sharply and reached a maximal level at 31.3° C. Activity fell sharply with falling colonic temperature thereafter (Fig. 5B). Maximal activity occurred at a lower colonic temperature for these rats than for the PTU-treated rats of experiment 3. Initial colonic temperature of these rats was also lower than that of PTU-treated rats but maximal activities of both groups occurred when colonic temperature had fallen 3 to 4° C. Initial colonic temperature of control rats in experiment 4 was also lower than counterparts in experiment 3 but maximal activities occurred in both experiments when colonic temperature had fallen only 0.5 to 1.0° C below initial temperature values. No explanation can be advanced for the lower initial colonic temperatures of both groups of rats in this experiment as compared with similar groups in experiment 3.

Discussion

The common notion that the activity level of rats is directly related to level of secretion of thyroid hormone does not appear to be correct. The spontaneous running activity of male rats kept in air at 25° C is not reduced by hypothyroidism as produced by propylthiouracil administration, but may even be increased slightly. The failure to observe a decrease in activity level of PTU-treated rats agrees with the results of Hoskins (12), Lee and Van Buskirk (10), and Hall and Lindsay (11) although these investigators used surgically thyroidectomized rats. Further, Lee and Van Buskirk (10) and Hall and Lindsay (11) failed to show a good correlation between metabolic rate and spontaneous running activity in their rats. On the other hand, the careful studies of Richter (8), also using surgically thyroidectomized rats, suggest that complete thyroidectomy is accompanied by a decrease in spontaneous running activity. Only those rats in whom a minute amount of thyroid tissue was found at autopsy maintained a normal activity level. He attributed results of experiments of others cited above to be due to minute amounts of thyroid tissue

remaining after thyroidectomy. Richter also reported that addition of small amounts of thyroid extract (1 to 2 grains/kg food) to the diet returned the depressed activity levels to normal. Lee and Van Buskirk (10) and Wang (20), using larger amounts of thyroid extract, reported results to the contrary, that is, that replacement therapy depressed running activity of thyroidectomized rats. The argument that minute amounts of functioning thyroid tissue remained could not be used in the case of PTU treatment since this drug inhibits both thyroidal and extrathyroidal sites of thyroid hormone production. In addition, the rats used here had enlarged thyroid glands and anemia, both of which are characteristic of the hypothyroid state induced by PTU (15). The studies of Mann (9), in which administration of thiouracil is reported to decrease spontaneous running activity of female rats in air at 25° C, are at variance with the results reported here. The difference may be related to the relatively greater toxicity of thiouracil than propylthiouracil. The former drug was used at the same dose level as the latter. A greater toxicity is suggested by the fact that Mann observed administration of thiouracil to disrupt the estrus cycle of his rats. It is our experience that this is not observed with the dose of propylthiouracil used here.

When male PTU-treated rats were exposed to cold (8° C), activity level increased during the first day of exposure. Untreated male rats also increased their activity under these conditions. This suggests that administration of PTU to rats did not prevent manifestation of the characteristic increase in running activity accompanying cold exposure. Activity level of PTU-treated rats decreased after the first day of cold exposure possibly because body temperature was falling.

Acute exposure to cold decreased body temperature of either PTU-treated or radioactive iodine-treated rats at a more rapid rate than that of control rats. The latter had a fall of colonic temperature amounting to only 2 to 3° C during the course of the experiment. Colonic temperatures stabilized at 34 to 36° C and were maintained by gradation of activity in which fall of colonic temperature was followed by an increase in activity until colonic temperature rose. In similar experiments on intact rats, Bartlett *et al.* (17) interpreted their results as suggesting that the rats which were most active during cooling cooled fastest. Thus, increased activity initiated the fall in body temperature. They did not measure colonic temperature continuously but at intervals of 1 hour during cooling. The results presented in Fig. 6 for a typical control rat during a portion of an experiment suggest the opposite interpretation, i.e., activity was adjusted to maintain body temperature. When colonic temperature falls, activity level appears to increase until temperature either rises or becomes stable. Thereafter, activity level falls until colonic temperature again falls. In this fashion mean colonic temperature of the group could be maintained at levels of 34 to 36° C for several hours.

Activity levels of both groups of control rats used in experiments 3 and 4 were maximal when colonic temperature had fallen 0.5 to 1° C. In the case of

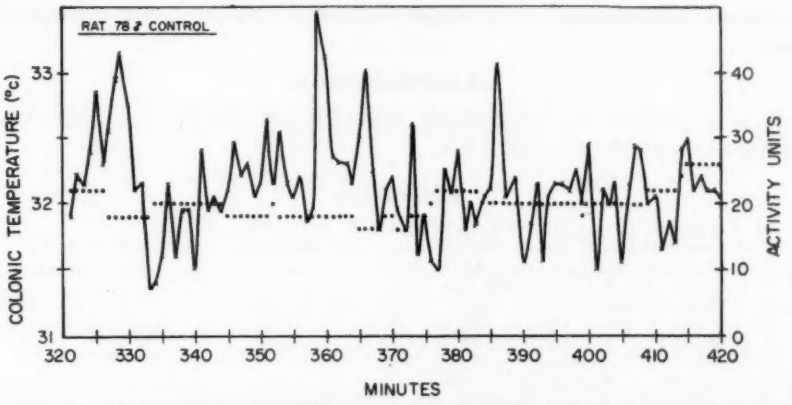

FIG. 6. Colonic temperature (●) and activity (×) of a typical control rat during a portion of an experiment. When colonic temperature falls, activity level appears to increase until colonic temperature either rises or becomes stable. Thereafter, activity level falls until colonic temperature again falls and activity level rises. In this fashion colonic temperature was maintained at about 32° C for 100 minutes. Activity and colonic temperature are shown for each minute.

hypothyroid rats, maximal activity was recorded only when colonic temperature had fallen 3 to 4° C. Apparently this increase in activity level when heat deficit was large could not counteract the fall in body temperature and the rats continued to cool until they were returned to a warm environment. Activity began too late. Reasons for this difference in activity pattern between control and thyroidectomized rats are not apparent. The results suggest, however, that regulation of body temperature by change of activity level under these conditions is an important aspect of total regulatory mechanisms contributing to maintenance of body temperature.

Response of hypothyroid rats to acute cold was similar to counterparts exposed more chronically in wheel-type activity cages. Both groups showed an initial increase in activity when cold exposure began. Further comparison is difficult because colonic temperatures of the hypothyroid rats in wheel activity cages were not measured. Perhaps increase in activity of the latter also began too late to be of much benefit in maintenance of body temperature.

The results presented here suggest that a relationship exists between colonic temperature and activity during cooling. It is recognized that this relationship may be only fortuitous. Activity level may be more closely related to skin temperature than colonic temperature. Indeed, recent studies have shown that mean skin temperature of hypothyroid rats exposed acutely to cold air is higher at a given colonic temperature during cooling than that of control rats (21). If these data can be considered applicable to the present experiments, then maximal activities of both hypothyroid and control rats occur during cooling at skin temperatures that are only about 1 Centigrade degree different. Further studies need to be done to determine whether either skin temperature

or colonic temperature is more closely related to activity during exposure to cold.

Acknowledgments

It is a pleasure to acknowledge the technical assistance of Mr. Paul Richardson and Mr. Howard Clark.

References

1. M. J. FREGLY. Can. J. Biochem. and Physiol. **37**, 425 (1959).
2. C. P. LEBLOND and J. GROSS. Endocrinology, **33**, 155 (1943).
3. B. H. ERSHOFF. Endocrinology, **43**, 36 (1948).
4. G. C. RING. Am. J. Physiol. **125**, 244 (1939).
5. E. A. SELLERS and S. S. YOU. Am. J. Physiol. **163**, 81 (1950).
6. M. J. FREGLY. Am. J. Physiol. **187**, 297 (1956).
7. L. G. BROWMAN. J. Exptl. Zool. **94**, 477 (1943).
8. C. P. RICHTER. Endocrinology, **17**, 73 (1933).
9. C. W. MANN. J. Psychol. **20**, 91 (1945).
10. M. O. LEE and E. F. VAN BUSKIRK. Am. J. Physiol. **84**, 321 (1928).
11. V. E. HALL and M. LINDSAY. Endocrinology, **22**, 66 (1938).
12. R. G. HOSKINS. Endocrinology, **11**, 97 (1927).
13. R. A. FISHER. Statistical methods for research workers. 10th ed. Hafner, New York. 1948. p. 114.
14. D. C. JONES, D. J. KINNELDORF, D. O. RUBADEAU, and T. J. CASTANERA. Am. J. Physiol. **172**, 109 (1953).
15. M. J. FREGLY, M. I. BAKER, and J. F. GENNARO, JR. Am. J. Physiol. **198**, 4 (1960).
16. E. F. ADOLPH, J. N. BARKER, and P. HOY. Am. J. Physiol. **178**, 538 (1954).
17. R. G. BARTLETT, JR., V. C. BOHR, G. L. FOSTER, M. S. MILLER, and R. H. HELMENDACH. Proc. Soc. Exptl. Biol. Med. **92**, 457 (1956).
18. M. J. FREGLY, J. F. GENNARO, JR., and A. B. OTIS. Circulation Research, **8**, 749 (1960).
19. M. W. PARROTT, P. W. DURBIN, and J. J. BURG. Proc. Soc. Exptl. Biol. Med. **98**, 404 (1958).
20. G. H. WANG. Bull. Johns Hopkins Hosp. **40**, 304 (1927).
21. M. J. FREGLY, P. F. IAMPIETRO, and A. B. OTIS. J. Appl. Physiol. **16**, 127 (1961).

NOTES

PASSAGE OF SALICIN AND SALIGENIN ACROSS THE WALL OF THE RAT ILEUM

V. W. Adamkiewicz and A. A. Fortier

While investigating the absorption of glycosides we observed that (*a*) the glycoside salicin passes across the wall of a surviving ileum several times more slowly than does the aglycone saligenin, and that (*b*) the glycoside does not lose its glucose during the passage.

Groups of 10 Sprague–Dawley, 150 g, male rats were used. The terminal portion of the ileum (20 cm) was quickly removed (ether anesthesia), flushed aborally with a solution of "Tyrode minus glucose" (T.M.G.), and its aboral end was ligated. The ileum was filled with 0.5 ml of T.M.G. containing either 5% w/v of the glycoside salicin (K. and K. Laboratories),* or 5, 0.5, and 0.1% w/v of the aglycone saligenin (K. and K. Laboratories). The oral end was then ligated. The ileums were placed each in a beaker with 14 ml of T.M.G., and incubated in a Dubnoff apparatus, at 36° C, 50 excursions per minute, in a O_2/CO_2 atmosphere, for 10 minutes. The total salicin which passed into the incubation medium was determined by the anthrone reaction (1), which measures the glucose content of the glycoside. The total saligenin was determined by a colorimetric reaction for phenols (2). The values were expressed in micrograms per milligram of dry ileum (24 hours at 70° C).

Saligenin passes across the ileal wall about 3 times faster than does salicin, when each is administered in a 5% w/v solution (Table I). Saligenin induces anesthesia of mucous membranes when administered in concentrations higher

TABLE I

Substance	μg of substance which passed in 10 minutes across the wall of ileum, expressed per mg of dry ileum (means of 10 rats ± S.E.)
Salicin (glycoside)	
5% w/v	2.00 ± 0.19
Saligenin (aglycone)	
5% w/v	7.65 ± 0.36
0.5% w/v	4.34 ± 0.41
0.1% w/v	0.39 ± 0.05

than 2% w/v. But this could not account for the rapid passage across the ileum because when saligenin is administered in a 0.5% w/v solution it still passes several times faster than does salicin. At such low concentrations the anesthetic action of saligenin is negligible.

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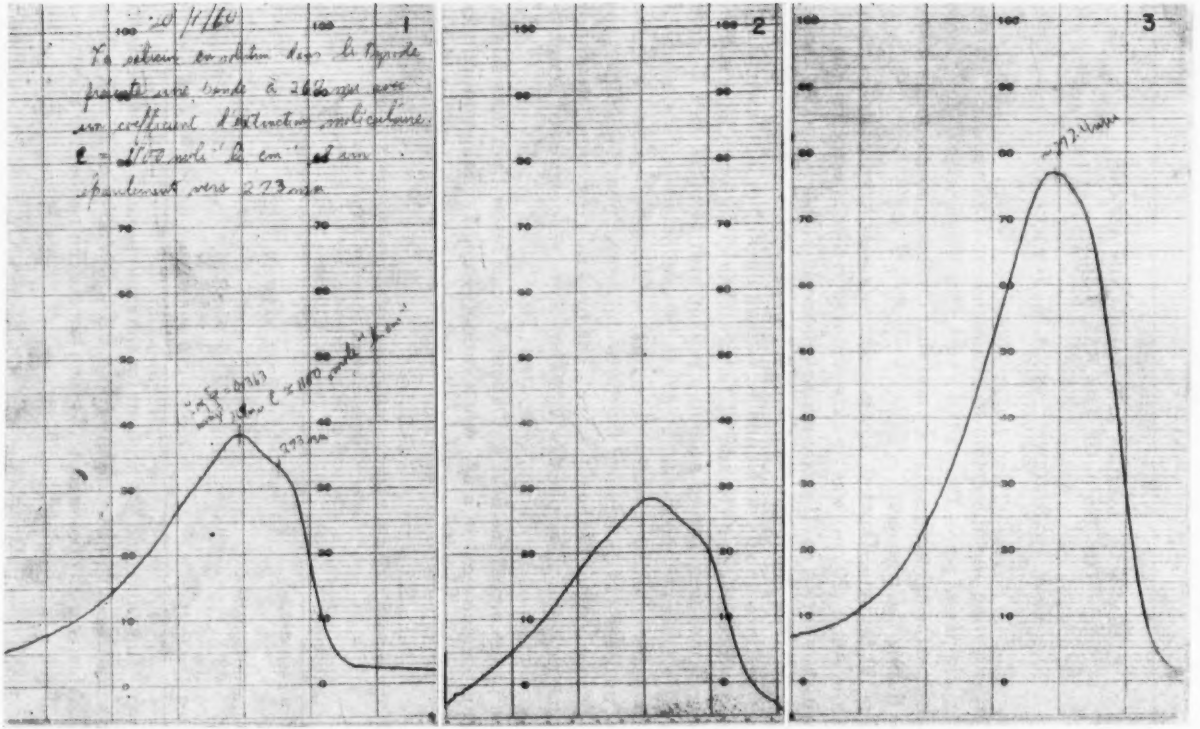

FIG. 1. Ultraviolet spectra of salicin and saligenin. (1) Pure glycoside salicin: peak at 267 mμ, shoulder at 273 mμ. (2) Salicin which passed across the ileal wall: peak at 267 mμ, shoulder at 273 mμ. (3) Pure aglycone saligenin: peak at 272 mμ, no shoulder. The height of the curves depends on the concentration of the substance. The irregularity of the base line is due to the incubation medium (see text) in which the substances are dissolved.

It was calculated that, in the case of salicin, the total amount of glycosidic glucose which passed into the incubation media, across the ileal wall, under the present experimental conditions, was 600 to 1000 μg. The incubation media were evaporated, the residues were dissolved in 4 ml of water, and the glucosazone test (3) was performed. It detects free glucose in a 0.01% w/v solution. All the tests were negative. But, 10 control tests performed on 4-ml samples of T.M.G., containing 600 μg of free glucose (0.015% w/v), all yielded glucosazones. It was concluded that at least 60% of salicin passed across the ileal wall without liberating free glucose.

Another lot of incubation media, into which salicin had passed across ileal walls, was investigated by ultraviolet spectroscopy. A peak was obtained at 267 mμ; E 11,000 mole^{-1}, liter cm^{-1}, and a shoulder at 273 mμ (Beckman D.K. 1 apparatus, 1-cm quartz cells). Identical spectra were obtained on 10 control samples of T.M.G. in which empty ileums had been previously incubated for 10 minutes, and to which pure salicin was added subsequently. But, when the pure aglycone saligenin was added, the spectra showed a peak at 272.4 mμ, and no shoulder (Fig. 1). It was concluded that salicin remains a glycoside during the passage across the wall of a surviving ileum. It may be added that insulin does not affect either the quantitative or the qualitative aspects of the present observations. A more complete report will be published elsewhere.

We wish to thank Professor C. Sandorfy and Mr. J. C. Patenaude, of the Department of Physical Chemistry, for the investigation of the ultraviolet spectra of salicin and saligenin.

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1. R. E. Semple. Can. J. Biochem. and Physiol. **35**, 383 (1957).
2. O. Folin and V. Ciocalteau. J. Biol. Chem. **73**, 627 (1927).
3. P. B. Hawk, B. L. Oser, and W. H. Summerson. Practical physiological chemistry. 13th ed. Blakiston Co. Inc., New York and Toronto. 1954. p. 63.

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EFFECT OF PROTEIN-FREE DIET ON THE VITAMIN A STORAGE AND DEPLETION IN THE RAT

T. K. Murray

Rechcigl *et al.* (1) recently reported that rats fed a "protein-free" diet stored twice as much vitamin A from a small daily intake as did rats fed an 18%

casein diet. Arnich and Pederson (2), however, found that while the utilization of carotene was increased by increasing dietary protein from 11% to 40%, the storage of preformed vitamin A was not affected. McClaren (3) indicated that protein depletion delayed the development of vitamin A deficiency symptoms in rats and suggested that liver stores of the vitamin were used up less quickly in the protein-deficient rat. On the other hand, Esh and Bhattacharya (4) reported that suboptimal levels of protein caused an increased disappearance of liver vitamin A. Because of these divergent results the following experiments were undertaken.

In the first experiment, weanling male rats of the Wistar strain were fed a "protein-free" or a "protein" diet for 2 weeks. The diets were similar to those used by Rechcigl *et al.* (1) except that starch and sucrose were used rather than glucose and dextrin and the casein was increased from 18% to 20%. Vitamin A or β-carotene was given orally, 3 times per week, at 100 I.U./dose. After 2 weeks the rats, 10 per group, were killed and their livers and kidneys assayed for vitamin A by the method of Ames *et al.* (5). The results are shown in Table I.

TABLE I

Growth and storage of vitamin A in rats fed a protein-free or a 20% protein diet

	Diet					
	Protein-free			20% protein		
		Vitamin A			Vitamin A	
Dose	Wt. gain, g	I.U./liver	I.U./kidney pair	Wt. gain, g	I.U./liver	I.U./kidney pair
Vitamin A	−6	376	2	30	351	6
Carotene	−5	73	2	36	66	14

Rats fed the "protein-free" diet and vitamin A lost an average of 6 g while those given protein gained 30 g. Similar weights were recorded for rats given β-carotene. Liver storage of vitamin A was not significantly affected by dietary protein whether the vitamin was administered as preformed vitamin or as β-carotene. When protein was fed, rats stored 2 to 7 times more vitamin A in their kidneys than when no protein was given. This is not an unexpected result as it has been noted previously (6, 7) that any restriction in growth results in low storage of vitamin A in the kidney. In any case the total amount of vitamin A involved was very small.

In this laboratory it is routinely found that 50%–60% of even small doses of vitamin A appear in the liver and this has not been altered by the lack of dietary protein. Rechcigl *et al.* (1) reported 43% storage of their 30 I.U./dose when protein was fed but almost 90% storage when no protein was given. This latter value appears to be unreasonably high. Our results with carotene do not agree with those of Arnich and Pederson (2) but they are hardly comparable; their much larger doses of carotene were given in water, while ours were made in oil.

In a second experiment the liver vitamin A of young male rats was increased with a single oral dose of vitamin A acetate. Two days later 10 aminals were killed and their liver vitamin A was determined. The remaining rats were fed the "protein-free" or "protein" diet. Seven rats from each group were killed after 14 days and in 28 days the remaining eight in each group were killed. The vitamin A content of all livers and of the kidneys in the last group was measured and the results are shown in Table II. Once again growth rates were

TABLE II

Growth and depletion of vitamin A stores in rats fed a protein-free or a 20% casein diet

	Diet					
	Protein-free			20% casein		
		Vitamin A			Vitamin A	
	Wt., g	I.U./liver	I.U./kidney pair	Wt., g	I.U./liver	I.U./kidney pair
Initial	105	3480		105	3480	
14 days	98	3020		130	3070	
28 days	85	2950	3	150	2840	5

greatly different but liver vitamin A levels not significantly so. It is interesting that the losses of vitamin A in the first 14-day period were about 30 I.U./day for both groups while in the next 14 days the group fed the "protein-free" diet lost only 5 I.U./day while the protein-fed group lost 8 I.U./day. More vitamin A appeared in the kidney when protein was fed but again the total amount was very small.

The results reported here support the statement of Rechcigl *et al.* (1) that protein is not essential for the storage of vitamin A in the liver but unlike these authors we did not find greater storage in rats fed no protein. Over a 4-week period the total loss of vitamin A from the liver was not significantly altered by the absence of dietary protein or by the resulting halt in growth.

1. M. Rechcigl, S. Berger, J. K. Loosli, and H. H. Williams. Nature, **183**, 1597 (1959).
2. L. Arnich and D. J. Pederson. Federation Proc. **15**, 212 (1956).
3. D. S. McLaren. Brit. J. Opth. **43**, 234 (1959).
4. G. C. Esh and R. K. Bhattacharya. Symposium on Proteins, Mysore, India. August 1960.
5. S. R. Ames, H. A. Risley, and P. L. Harris. Anal. Chem. **26**, 1378 (1954).
6. R. M. Johnson and C. A. Baumann. J. Nutrition, **35**, 703 (1948).
7. T. K. Murray, J. L. Beare, and J. A. Campbell. Can. J. Biochem. and Physiol. **38**, 663 (1960).

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CORRECTIONS

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Page 644. In the first paragraph under "Procedure" the last sentence should read:
"Immediately before the flame photometry 1 ml of ethanol solution A was added to each of tubes Nos. 1 and 2."

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CONTENTS

Page

BIOCHEMISTRY

Guy N. Emery and J. M. R. Beveridge—The cause of the disappearance of arginine from the blood of rats with acute hepatic necrosis induced by dietary means 977

Dorothy S. Dow and C. E. Allen—Steady-state oxidation of glucose in hyperthyroid and hypothyroid rats 981

G. E. Connell and R. W. Shaw—The purification of haptoglobin 1013

H. Tsuyuki and D. R. Idler—The metabolism of inositol in salmon. III. The biochemical reactions of 2-C^{14}-myoinositol in coho liver 1037

D. K. Myers, C. Anne Hemphill, and Constance M. Townsend—Deoxycytidylate deaminase levels in regenerating rat liver 1043

J. E. Smith and E. R. Waygood—Resolution of the apoenzyme and coenzyme of L-glutamic acid decarboxylase from sunflower cotyledons 1055

A. Froese and A. H. Sehon—Problems involved in the purification of antihapten antibodies by the use of stroma–hapten conjugates 1067

J. M. Neelin and W. H. Cook—Terminal amino acids of egg yolk lipoproteins . 1075

Notes:

T. K. Murray—Effect of protein-free diet on the vitamin A storage and depletion in the rat. 1099

PHYSIOLOGY

D. V. Bates and E. V. Harkness—Notes on the application of the Hersch galvanic oxygen cell to measurement of blood oxygen content and tension 991

R. M. Green, K. G. McNeill, and G. A. Robinson—The distribution of potassium and caesium-137 in the calf and pig 1021

Frank C. Monkhouse, Jean Strachan, and Florence McClain—Studies on clearing factor lipase (CFL) of postheparin plasma 1027

W. F. Perry and Helen F. Bowen—The in vitro utilization and production of non-esterified fatty acids by adrenalectomized rats 1061

Melvin J. Fregly—Effect of changes of ambient temperature on spontaneous activity of hypothyroid rats 1085

Notes:

V. W. Adamkiewicz and A. A. Fortier—Passage of salicin and saligenin across the wall of the rat ileum 1097

PHARMACOLOGY

W. C. Stewart and D. H. McKay—Some respiratory and cardiovascular effects of gradual sarin poisoning in the rat 1001